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Drug Resistance Reversal, Anti-mutagenicity and Antiretroviral Effect of Phthalimido- and Chloroethyl-Phenothiazines

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Abstract. *The effect of substituted phenothiazines was studied in three different systems; bacteria and cancer cells and reverse transcriptase enzyme of Moloney leukemia virus. F'lac and hemolysin plasmids were eliminated by some substituted phenothiazines from E. coli at a very low frequency. The same phenothiazine derivatives also were synergistic with tetracycline in bacteria and shown antimutagenic effect in Ames test. No mutagenic effects were observed in TA 98 strain of Salmonella typhimunium. Chloroethyl-substituted phenothiazines showed antimutagenicity equivalent to the parent compounds; however, phthalimido-substituted phenothiazines had higher antimutagenicity of 50%. P-glycoprotein responsible for multidrug resistance was also inhibited in tumor cells. The accumulation of the fluorescent rhodamine 123 in the phenothiazine treated multi-drug resistant tumor cells was measured by flow cytometry. Some of the substituted phenothiazines were effective P-glycoprotein blockers, while some compounds had moderate activity, but others were without effect as compared to 5 μM verapamil. On the basis of computer analysis there are some correlations between the biological activities and the dipole moments, and entropy of the studied molecules. Our results suggest that the inhibition of Hly$^+$ plasmid replication and P-glycoprotein function may depend partly on similar electronic properties of the studied phenothiazine derivatives. The activity of Moloney leukemia virus reverse transcriptase was inhibited by the substituted phenothiazines, however, no basic differences were found in the activities of phthalimido- and chloroethyl substituted phenothiazines.*

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Resistance to chemotherapeutics has recently been observed to be emerging among viruses, bacteria, fungi, protozoa and cancer cells. The basic reason for the development of this resistance is Darwinian selection, which ensures the accommodation of the living organisms to an altered environment. The genetic basis for drug resistance can be extrachromosomal plasmids in bacteria and chromosomal mutations. The former plasmid makes the living organism more flexible in its adaptation to the environmental changes, ensuring the evolution of resistance by intercellular transfer of plasmids. The latter is less frequent and mutations are more conservative than plasmid encoded DNA and those can be tested in mutagenicity assays. The inhibition of mutations can result in a chemopreventive effect in cancer induction or resistance development in bacteria and cancer cells, in addition the inhibition of ABC transporter related effect pumps of multidrug-resistant bacteria and cancer cells can restore the sensitivity of resistant cells as well. Such efflux pumps in bacteria and cancer cells reduce the effective drug concentration inside the cells to an ineffective level.

P-glycoprotein sequences of multidrug resistant cancer cells have a substancial degree of sequence similarity with a family of bacterial transporter proteins, *i.e.,* hemolysin (1). On the basis of the homology between bacterial and cancer cell-produced ABC transporters, the consequences of hemolysin plasmid elimination and *mdr* efflux inhibition were compared.

This paper reports the reversal of similar resistance mechanisms in bacteria and cancer cells, *via* inhibition of the drug efflux mechanisms by certain phenothiazines. The mutagenic and antimutagenicity studies were planned to give evidence for the effects on chromosomal DNA or for possible

Table I. *Effect of phenothiazines (1, 2, 3, 4) on L-5178 cells with multidrug resistance.*

Compound's number	$(CH_2)n$	R	Concentration (M)	Forward scatter height [cell size ratio]	Side scatter height [granulation of cell ratio]	Fluorescence one height[a] x^b	$\frac{x}{256}$	$y=10^{\frac{x}{256}}$	Fluorescence activity ratio[c]
10-[N-(Phthalimido)alkyl]-2-substituted-10H-phenothiazines									
[1]	3	Cl	1×10^{-5}	541.63	215.98	615.46	2.40	253.59	10.65
			2×10^{-5}	538.05	219.75	704.47	2.75	564.72	23-72
[2]	4	Cl	1×10^{-5}	535.16	220.48	595.62	2.32	212.14	8.91
			2×10^{-5}	537.95	222.88	657.17	2.56	369.03	15.50
1-(2-Chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkyl-1-ureas									
[3]	4	Cl	1×10^{-5}	535.93	210.71	520.21	2.03	107.66	4.52
			2×10^{-5}	534.05	211.90	633.59	2.47	298.51	12.54
[4]	3	CF_3	1×10^{-5}	535.31	216.00	538.84	2.10	127.30	5.34
			2×10^{-5}	532.75	226.20	599.29	2.34	219.26	9.21
(+)verapamil[d]			4 μg	545.74	216.62	451.14	1.76	57.84	2.43
DMSO[e]			10 μL	145.33	210.62	316.49	1.23	17.23	0.72
par[f]			control	504.86	184.75	732.17	2.86	724.50	30.44
mdr + R123[g]			control	552.95	211.52	352.41	1.37	23.80	1.00

[a] Ref 12. [b]x: Measured fluorescence value at linear scale [mg, uptake of R123].
[c]The R-123 accumulation was calculated from fluorescence of one height value using 1st equation $y=10^{\frac{x}{256}}$ then the fluorescence activity ratios were calculated according to the formula given below;

$$\text{Ratio} = \frac{\text{(mdr treated/mdr control)}}{\text{(parental treated/parental control)}}$$

[d](±)verapamil: a control for mdr reversal; [e]DMSO (dimethylsulfoxide): solvent control;
[f]par parental without multidrug resistant gene; [g]mdr parental with multidrug resistant gene.

cancer chemoprotective effects. The mutagenic, antimutagenic and antiretroviral effects of some phthalimido- and chloroethyl-substituted phenothiazines will be studied and reported in the hope that new chemotherapeutics can be developed.

Materials and Methods

Chemicals. Four phenothiazine derivatives, *i.e.,* 10-[3-(phthalimido) propyl]-2-chloro-10*H*-phenothiazine [*1*], 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [*2*], 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [*3*]and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [*4*], used in this paper were prepared as described previously [*2*]. Phenothiazine [*5*] and promethazine [*6*] were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chlorpromazine [*7*] was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). (±)-Verapamil (Sigma, V 4629) as calcium channel blocker (3,4) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rhodamine 123 hydrate (R123)(Aldrich, 28394-0) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Tissue culture. L5178 mouse T cell lymphoma and its MDR1/A retrovirus transfected derivative were provided by Prof. Aszalos.

Bacterial strains. *E. coli* JF 2571/Rm98 was resistant to ampicillin, streptomycin, sulphonamide and tetracycline. Haemolysin producing bacteral isolates were obtained from clinical specimens and two laboratory strains of *E. coli* CW-22 and CW-35 were used in the studies (kindly provided by L. Emôdy, Institute of Microbiology, POTE, Pécs,

Hungary). The bacteria were grown in minimal tryptone yeast extract broth or agar (MTY) as described by Alföldi *et al* (5). The TA 98 strain of *Salmonella typhimunium* was obtained from Prof. Bruce Ames. The reverse transcriptase of Moloney leukemia virus was a recombinant enzyme produced by New England BioLabs.

Plasmid curing effect of phenothiazines. Plasmid elimination was carried out as describd earlier (6,7). An overnight preculture of *E. coli* JF 2571/Rm98 was diluted 10^{-4} fold and 50 µL (about 5×10^3) bacteria was inoculated of this dilution into 5.0 mL MTY both. Increasing concentrations of the tested drugs were added and the tubes were incubated at 37°C for 24 hours. From the tubes showing growth, different dilutions were prepared and 0.1 mL samples were plated on MTY and blood agar plates. The plates were incubated at 37°C for 24 hours then colonies were counted (master plate). The isolated colonies were replicated with Lederberg's velvet replica plating (8) into MTY plates containing tetracycline (5 µg/mL). After incubation at 37°C for 24 hours the number of colonies was compared to the number of colonies growing on the master plate.

Synergism between phenothiazines and tetracycline. The synergism between phenothiazines and tetracycline was performed on hemolysin producing and non-hemolytic *E. coli* strains. Tetracycline hydrochloride 36 µg/mL was mixed with MTY agar and poured into Petri dishes, which contained a filter paper strip 5 × 50 mm (imersed into the phenothiazine solutions 2500 µg/mL). After solidifying of the nutrient agar, the hemolysin positive and negative bacterial cultures were inoculated as a line perpendicularly to the filter paper. Inocubation was 37°C for 16 hours then synergism was evaluated.

Cell and fluororescence uptake. MDR1/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype (9). The L5178 MDR cell line, and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquot to Eppendorf centrifuge tubes. Then, the tested compounds were added in 2.0 mL of the 2.0 mg/mL stock solution and the samples were incubated for 10 minutes at room temperature. Then, 50 µL rhodamine 123 (R123) as an indicator was added to the samples (5.2 µM final concentration) and the cells were incubated for further 20 minutes at 37°C, washed twice and resuspended in 0.5 µl phosphate-buffered saline (PBS) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument (cell sorter). (±)Verapamil was used as a positive control in the R123 accumulation experiments (10,11). The R123 accumulation was calculated from $_x$fluorescence of one height value using the 2nd equation $y=10^{\frac{x}{256}}$ (Table I). In the case of logarithmic transformation, the 1024 digital channels are switched to one decade at each 256 ($=2^8$) channels. Then, the percentage of control mean of the fluorescence intensity was calculated for parental and mdr cell lines, compared to untreated cells. An activity ratio was calculated by the following equation (10,12)(Table I):

$$\text{Ratio} = \frac{(mdr \text{ treated}/mdr \text{ control})}{(\text{parental treated}/\text{parental control})}$$

Mutagenicity assay. Spot test was performed as described previously (13). The bacterial strain was cultured in Oxoid nutrient broth No.2 for 16 hours for turbidity to reach the optical density of 0.5 at 600 nm. After 0.1 mL of bacterial culture and 0.1M sodium phosphate buffer (pH 7.4) were mixed and overlaid onto minimal glucose agar, 9 mm in diameter well was made in the center of Petri dish and 1mL of 5 mg/mL test chemical was applied. All tested chemicals were dissolved in DMSO. 10 µg/mL 4-nitro-1,2[-phenylenediamine was used for positive control. After 48 hours, the presence of background lawn on all plates was confirmed and the number of back mutant colonies around the well were counted. The assay was performed in duplicate in two separate occasions.

Table II. *The determination of antibacterial and antiplasmid effects of some phenothiazines.*

Compound's number	$(CH_2)n$	R	Concentration (µg/mL)	Plasmid elimination (%)	MIC µg/mL (MIC)[1)]
10-[N-(Phthalimido)alkyl]-2-substituted-10H-phenothiazines					
[1]	3	Cl	160	0	
			200	0	>200
[2]	4	Cl	160	0	>200
			200	0	
1-(2-Chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkyl-1-ureas					
[3]	4	Cl	80	0	
			100	0	150
[4]	3	CF_3	160	0	
			200	0	>200
Phenothiazines					
[5]			160	0	
			200	0	>200
[6]			40	2.4	90
			60	14.9	
			80	85.3	
[7]			40	3.2	
			60	15.0	
			80	26.0	85

[1)] Minimal inhibitory concentration

Anti-mutagenic assay. TA 98 strain of *Salmonella typhimurium* was obtained from Prof. Bruce Ames. The plate incorporation test was performed as described previously (13) with some modifications for anti-mutagenicity assay. The bacterial strain was cultured in Oxoid nutrient broth No.2 for 14-16 hours until turbidity reached the optical density of 0.3 at 600 nm. 0.1 mL of bacterial culture, 0.1 mL of phenothiazines and 0.5 mL of 0.1M sodium phosphate buffer (pH 7.4) were mixed and incubated for 20 minutes at 37°C in a turbulating incubator, then 0.1 mL

Table III. *The determination of mutagenicity and anti-mutagenicity of some phenothiazines*[1,3].

Compound's number	$(CH_2)n$	R	Mutagenicity (spot test)	Anti-mutagenicity (%)
10-[N-(Phthalimido)alkyl]-2-substituted-10H-phenothiazines				
[1]	3	Cl	0	50
[2]	4	Cl	0	48
1-(2-Chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkyl-1-ureas				
[3]	4	Cl	0	16
[4]	3	CF_3	0	20
phenothiazines				
[5]			0	12
[6]			0	33
[7]			0	24
positive control				
4-nitro-1,2-phenylenediamine (mutagenicity)			+	–
chlorpromazine (antimutagenicity)	3	Cl	–	24

Table IV. *The synergistic effects of some phenothiazine with tetracycline on haemolytic and non-haemolytic E. coli strains.*

Compound's number	$(CH_2)n$	R	Synergism with tetracycline[1]	
			haemolytic *E. coli*	non-haemolytic *E. coli*
10-[N-(Phthalimido)alkyl]-2-substituted-10H-phenothiazines				
[1]	3	Cl	–	w
[2]	4	Cl	–	w
1-[2-(Chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkyl-1-ureas				
[3]	4	Cl	w	+
[4]	3	CF_3	–	w
phenothiazines				
[5]			–	–
[6]			+	+
[7]			+	+

[1] +: synergism.; –: no-synergism.; w: weak synergism.

of 10 μg/mL of 4-nitro-1,2-phenylenediamine was added for the mixture and incubated for 20 minutes at 37°C in a turbulating incubator. The mutagenicity of the 4-nitro-1,2-phenylenediamine derivative was compared in the presence of the compounds. The samples were poured into minimal agar and after 48 hours incubation, the mutant colonies were counted considering in the presence of background growth. Anti-mutagenicity assay was performed in triplicate in two separate occasions.

Quantum-chemical calculations. The molecular orbital calculations by parametric method 3 (PM3) were performed with the application of MOPAC program (Version 6.01) (14). The geometries of the neutral species [**1, 2, 3, 4, 5, 6, 7**] were optimized with respect to all geometrical parameters using Broyden-Fletcher-Goldfarb-Shanno algorithm incorporated in the program. For these calculations, the FACOM M770 computer in the Josai University Information Sciences Center was used.

Antiretroviral effect. Moloney murine leukemia virus (M-MuLV) reverse transcriptase assay. Test compounds and 3'-azido-3'-deoxythymidine triphosphate (AZT-TP) were assayed for their ability to inhibit recombinant M-MuLV reverse transcriptase (RT). The assay was performed as follows; the poly(rA)$_n$oligo(dT)$_{12-18}$ directed incorporation of [^{3}H]dTTP (Amersham) into cDNA. The 10x reverse transcriptase buffer contains 500 mM Tris-HCl (pH 8.3), 80 mM $MgCl_2$, 300 mM KCl, and 100 mM DTT. In all experiments the final volume of reaction assay was 20 mL. This contained water, 2 μL of 10x buffer, 20 μg/mL template-primer, 5 μM dTTP precursor, 0.2 μCi tritiated precursor, compounds tested (administered into the medium before adding the enzyme) and 5U reverse transcriptase initiating the reaction. This procedure was followed by an incubation for 40 min at 37°C. 15 μL of the mixture was

Table V. *Calculated dipole moments, π-LUMO, π-HOMO energies, and distance between N10 and nitrogen atom on seven compounds [1, 2, 3, 4, 5, 6, 7] by PM3 method.*

Compd's number	$(CH_2)n$	Dipole moment (Debye unit)	π-LUMO (eV unit)	π-HOMO (eV unit)	d[1]	Antitumor activity[2]
[1]	3	3.31	-1.188	-7.997	5-04	
[2]	4	4.31	-1.195	-7.982	6.33	
[3]	4	2.92	-0.549	-8.162	6.30	
[4]	3	4.34	-0.972	-8.433	5.00	
[5]	0	2.42	-0.298	-7.968		–
[6]	2	2.88	-0.265	-8.012	3.79	–
[7]	3	1.07	-0.447	-8.045	5.08	+

[1] Distance between N10 and nitrogen atom (in Å units). [2] Ref: 17; Antitumor activity: +, positive: -, negative.

then transffered to a Whatman DE 81 filter paper disc, washed by 5% dinatrium-hydrophosphate buffer, water, then 96% ethanol, and after drying and putting into 5 ml scintillation cocktail (OptiPhase 'HiSafe 3', Wallac) radioactivity was measured by Packard Tri-Carb 4530 liquid scintillation counter. The residual enzymatic activities were compared to the control (no drug added). The IC_{50} of AZT-AT was 0.2 μM in our experiments.

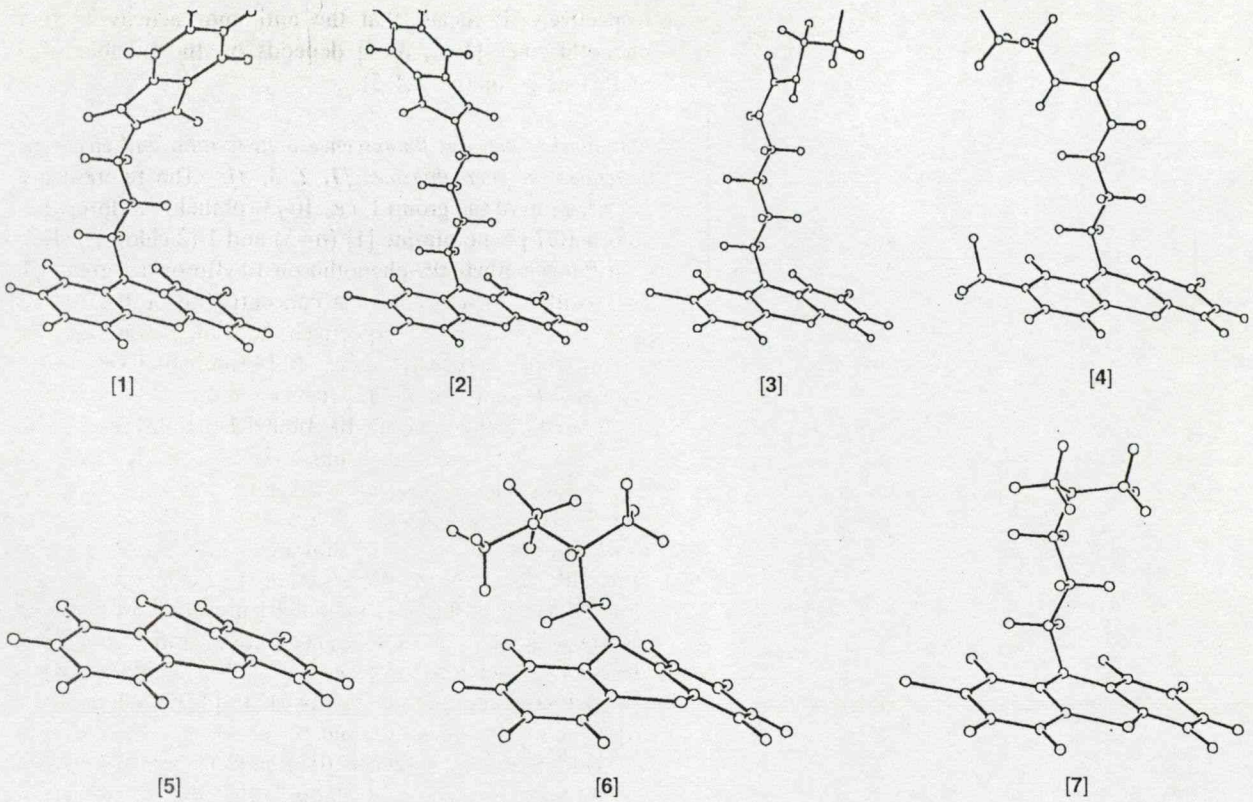

Figure 1. *The optimized structures of seven compounds [1, 2, 3, 4, 5, 6, 7] by PM3 method.*

Results and Discussion

The ring substitutions which were favourable for antitumor effect (Table I) resulted ineffective molecules in plasmid elimination (Table II). In addition, the MIC values were higher than 200 μg/mL for the *E. coli* strains tested. These data suggest that the substitution introduced into the phenothiazine reduced the cell wall permeability of the compounds. The compounds were also tested for mutagenic effects in the direct Ames test without microsomal fraction. As is shown in Table III, these phenothiazines were not mutagenic. Considering the lack of mutagenic activity of these phenothiazines [**1-7**] and the presence of antimutagenic, antiplasmid and synergistic effect with tetracycline, a search for structure-activity relationship was promising (Table IV). Anti-mutagenic activity and the antitumor effects of the derivatives were systematically studied and the effects were compared to the quantum-chemical properties of the substituted phenothiazines.

We calculated the dipole moments (in Debye unit), π-LUMO and π-HOMO, energies (eV unit), and the distance (Å unit) between 10th nitrogen on phenothiazine skeleton and terminal nitrogen of aliphatic chain group for the relationships between the electronic structures and antitumor activity (Table V). In all cases, the PM3 optimized geometries were slightly nonplanar, and structures are shown in Figure 1. It is interesting that substituted phenothiazines had different anti-mutagenic activity, however none exerted mutagenic effects. The reverse transcriptase enzyme inhibition of murine leukemia virus shows that the substituents introduced into the phenothiazines do not alter significantly the antiretroviral effect of phenothiazines. However, the reverse transcriptase inhibition makes a wider perspective for phenothiazines as anticancer compounds (Figure 2).

Relationship between distance from N1O to nitrogen atom with group, and antitumor activity: The distance of the group 1, *i.e.,* 10-[3-(phthalimido)propy]-2-chloro-10*H*-phenothiazine [**1**], (n=3), 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**], (n=3) and chlorpromazine [**7**], (n=3) with stronger compounds showed 5.04, 5.00 and 5.08Å, respectively. Second, the distances of group 2, *i.e.,* 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**], (n=4), 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**], (n=4) with weaker activity, phenothiazine [**5**], (n=0), promethazine (**6**, n=4) with non-antitumor activity showed 6.33, 6.30 and 0, 3.79Å, respectively (Table V).

Interestingly, it suggested that a direction of a lone electron

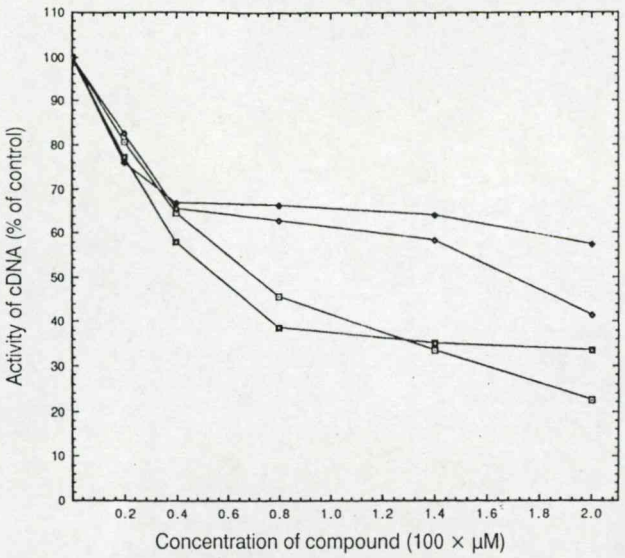

Figure 2. *The inhibition of Moloney leukemia virus reverse transcriptase by phenothiazine compounds. Activity of the control was 21500 CPM. 10-(4-phthalimido)butyl-2-chloro-10H-phenothiazine [2]:* ●, *1-(2-chloroethyl)-3-(2-trifluoromethyl)-10H-phenothiazin-10-yl)propyl-1-urea [4]:* ○, *1-(2-chloroethyl)-3-(2-chloro)-10H-phenothiazin-10-yl)butyl-1-urea [3]:* ■, *10-(3-phthalimido)propyl-2-chloro-10H-phenothiazine [1]:* □.

pair on 10th nitrogen of phenothiazine skeleton and the length between 10th nitrogen of phenothiazine skeleton and terminal nitrogen (3N or 4N) of methylene group might have a role on the activity of the compounds (Figure 1). The 3-(phthalimido)propyl group of 10-[3-(phthalimido)propy]-2-chloro-10*H*-phenothiazine [**1**], n=3), and a 1-(2-chloroethyl-1-urea)-3-propyl group of 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**], (n=3), and a 3-dimethylaminopropylene group of chlorpromazine [**7**], (n=3) have the bending tendency of orientation inside the phenothiazine skeleton (Figure 1). On the other hand, (phthalimido)butyl group of 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**], (n=4), or a 1-(2-chloroethyl-1-urea)-3-butyl group of 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**], (n=4), phenothiazine [**5**], and promethazine [**6**] have the orientation outside the phenothiaizne skeleton (Figure 1). However, pentamethylene derivative of phenothiazine (n=5) had again the inner orientation (data not shown). This is an apparent difference between group 1 compounds and group 2 compounds. This active conformation of group 1 compounds might be very convenient to attack the cytosine and guanine sites of DNA, or electrostatic P-glycoprotein membrane sites (15). Additionally, the proposed inner orientation can be supported by the C-C bonds of phenothiazine ring 1.390-1.405Å. The double bonds were 1.386-1.398Å except for the central bonds such as C4a-C10a with 1.402-1.408Å, the C9a-N10 and N10-C10a were 1.437-1.449Å and 1.437-1.450Å, respectively, and the C4a-S5 and S5-C5a were 1.755-1.760Å, respectively. It means that the antitumor activity of four phenothiazines [**1, 2, 3, 4**] depends on the number of a methylene group (n=3, 4, 5).

Relationship between fluorescence activity ratio and electronic structures on phenothiazines [1, 2, 3, 4]: The fluorescence activity ratio of the group 1, *i.e.,* 10-[3-(phthalimido)propy]-2-chloro-10*H*-phenothiazine [**1**] (n=3) and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**, n=3) with stronger activity at concentration of 1 x 10^{-5} M showed 10.65 and 5.34 , respectively. Second, the fluorescence activity ratio of group 2, *i.e.,* 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**] (n=4) and 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**] (n=4) with weaker activity showed 8.91 and 4.52, respectively (Table I). The relationship between multi-drug resistance reversal effects (fluorescence activity ratio) of phenothiazines at concentration of 2 × 10^{-5} M showed also the similar correlation with the concentration except 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**] (n=4). From the measurement of fluorescence activity ration by R123, among four phenothiazines, *i.e.,* 10-[3-(phthalimido)propy]-2-chloro-10*H*-phenothiazine [**1**] (n=3), 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**] n=4), 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**] (n=4) and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**] (n=3), a good relationship was found between multi-drug resistance reversed effect and fluorescence activity ration (Table I). However, a significant relationship between dipole moment, π-LUMO, or π-HOMO, and antitumor activity could not be found (Table V).

Moreover, four phenothiazines [**1, 2, 3, 4**] did not show any mutagenic effect but were antimutagenic in the Ames test. Therefore, the direct antitumor activity of phenothiazines (16,17,18) can possibly be exploited for experimental drug design. In this process, other beneficial biological effects like antimutagenic, antiviral (19), antiplasmid (6,7), reversal of multi-drug resistance in cancer cells (20) and synergism with antibiotics are considered as well.

We could therefore conclude from these results that electron relationship contributions are strongly related to a lone electron pair on the 10th nitrogen of the phenothiazine skeleton, and the length between the 10th nitrogen of phenothiazine skeleton and the terminal nitrogen (3N or 4N) of alkylene group. Additionally, we propose that the inhibition of the membrane-efflux mechanism using a reverse transcriptase enzyme of a retrovirus by phthalimidophenothiazines, or chloroethylphenothiazines can be exploited in the drug design for anticancer chemotherapy.

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References

1 (a) Gerlach JH, Endicott JA, Juranka PF, Henderson G, Sarangi F, Deuchars KL and Victor L: Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature *324:* 485-489, 1986. (b) Bradley G and Juranka PF, Ling V: Mechanism of multidrug resistance. Biochim Biophys Acta *948:* 87-128, 1988. (c) Gottesman MM and Pastan I: Biochemistry of multidrug mediated by the multidrug transporter. Ann Rev Biochem *62:* 385-427, 1993.

2 Motohashi N, Kawase M, Kurihara T, Hevér A, Nagy Sz, Ocsovszki I, Tanaka M and Molnár J: Synthesis and antitumor activity of 1-(2-chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkyl-1-ureas as potent anticancer agents. Anticancer Res *16:* 2525-2531, 1996.

3 Fleckenstein A: Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. Ann Rev Pharmacol *17:* 149-166, 1977.

4 Janis RA, Silver PJ and Triggle DJ: Drug action and cellular calcium regulation. Adv Drug Res *16:* 309-591, 1987.

5 Alföldi L, Rasko I and Kerekes E: L-Serine deaminase of *E. coli*. J Bacteriol *86:* 1512-1518, 1968.

6 Molnár J, Kiraly J and Mándi Y: Antibacterial action and R-factor inhiting activity of chlorpromazine. Experientia *31:* 444-445, 1975.

7 Mándi Y, Molnár J, Holland IB and Béládi I: Efficient curing of an *Escherichia coli* F-prime plasmid by phenothiazines. Genet Res Commun *26:* 109-111, 1976.

8 Lederberg J and Lederberg EM: Replica plating and indirect selection of bacterial mutants. J Bacteriology *63:* 399-406, 1952.

9 Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC: A retrovirus carrying an *MDR1* cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Natl Acad Sci USA *85:* 4486-4490, 1988.

10 Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S and Aszalos A: The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int J Cancer *54:* 456-461, 1993.

11 Kessel D: Exploring multidrug resistance using rhodamine 123. Cancer Commun *1:* 145-149, 1989.

12 Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J and Rosenberg SA: Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res *54:* 1766-1771, 1994.

13 Maron DM and Ames BN: Revised methods for the *Salmonella* mutagenicity test. Mutation Res *113:* 173-215, 1983.

14 (a) MOPAC ver. 6.00, J.J.P. Stewart, QCPE Bull *9:* 10, 1989. (b) Revised as ver. 6.01 by Yoshihisa Inoue, JCPE Newslett *4:* 60, 1992.

15 Rohrer DC: 3D molecular similarity modelling in computational drug design. *In:* Molecular Similarity and Reactivity. From Quantum Chemical to Phenomenological Approaches. (Carbó R ed). Understanding Chemical Reactivity vol.14. Dordrecht, The Netherlands, Kluwer Academic Publishers, 1995, pp141-161.

16 Molnár J, Mándi Y, Boda K and Földeák S: The effect of ring-substituted and quaternary chlorpromazine derivatives on the survival of the NK/Ly ascites tumor bearing mice. Neoplasma *29:* 215-222. 1982.

17 Motohashi N: Test for Antitumor activities of phenothiazines and phenoxazines (in Japanese). Yakugaku Zasshi *103:* 364-371, 1983.

18 Molnár J, Taródi B, Gálfi M, Matkovics B and Motohashi N: *In vitro* antiproliferative effects of tricyclic psychopharmacons and synergism with some resistance modifiers. Anticancer Res *12:* 273-280, 1992.

19 Mucsi I, Molnár J, Burián K, Béládi I, Sakagami H, Nakashima H, Inazawa K and Motohashi N: Antiviral activity of phenothiazine and benzo[*a*]phenothiazine derivatives. *In:* Biological and Chemical Aspects of Thiazines and Analogs, eds Jacques Barbe, H.Keyzer and Jean Claude Soyfer, Enlight Associates. pp 347-356, 1995.

20 Molnár J, Pusztai R, Hevér A, Nagy Sz and Motohashi N: Effects of two benzo[*a*]phenothiazines on multi-drug resistance (mdr) and tumor antigen expression. Anticancer Res *15:* 2013-2016, 1995.

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II.

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The Role of Stereoselectivity of Chemosensitizers in the Reversal of Multidrug Resistance of Mouse Lymphoma Cells

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Abstract. *The effect of three different stereoisomer pairs of CNS (central nervous system) active compounds was sudied on the activity of human mdr1 p-glycoprotein. The methotrimeprazine, clopenthixol and butaclamol isomers had an antiproliferative effect (ID_{50}) on the mdr1 expressing cells at o.250 μg/ml, while the parental cells were less sensitive having ID_{50} at 0.37-0.69 μg/ml. Enantiomers of methotrimeprazine and clopenthixol had similar effectivity on the drug efflux of mdr cells. However, (-)butaclamol was found to inhibit mdr efflux-pump activity much more than the CNS active (+) isomer. Based on these results,tricyclic compounds does not seem to have stereoselectivity in methotrimeprazine and clopenthixol on the mdr reversal effect. In general, both active and inactive members of stereoisomers had a similar effect on the drug accumulation of the mdr cells. Therefore, hypothetically the CNS inactive member of stereoisomer pairs can be used as a resistance modifier without any risk in patients suffering from drug resistant cancer.*

Multidrug resistance of cancer cells is associated with a large number of chemotherapeutic medicines due to the over expression of a membrane localized p-glycoprotein. This protein confers drug resistance through its action as a drug efflux-pump by reducing the intracellular concentration of anticancer drugs. Therefore, the medicines (chemicals) that block the action of mdr p-glycoprotein can be useful in treating cancer patients whose tumors are resistant to multiple chemotherapy.

In previous studies we investigated the effect of a large number of drugs and chemicals including antibiotics, psychopharmacons and beta-adrenergic antagonists on the action of efflux-pump. The most effective compounds were the promethazine, amitriptiline, propranolol and verapamil. While the verapamil isomers were effective in the same concentrations remarkable differences were found in the effect of two cyclosporin analogues (2,3).

A dissociation of the mdr reversal effect and cancer specific antiproliferative effect were found in some newly synthetised compounds.

Interestingly, two stereoisomers of three medicines, had practically the same effect on bacterial plasmid replication (5). Although their biological effects were entirely different in the central nervous system.

Therefore, we suggested that minor modifications in the chemical structure, such as changes in stereosymmetry could modulate the mdr reversing effects of methotrimeprazine, butaclamol and clopenthixol isomeres.

The effect of six stereoisomers was promising is the study of the action of mdr p- glycoprotein. In this paper we will review our recent studies on the structure activity relationships of tricyclic psychopharmacons as chemosensitizers, and present a working hypothesis on how the efflux pump activity is modulated in response to comparative toxicity of different substances in the CNS. The results suggest to us that non-toxic (CNS- ineffective) member of stereoisomeric pairs can reverse the multidrug resistance of cancer cells. The chosen drugs to reverse drug resistance has been compared to that of promethazine and verapamil.

Materials and Methods

Chemicals. Verapamil and promethazine were obtained from EGIS Works, Hungarian Pharmaceutical Company, Budapest. D-methotrimeprazine, L-methotrimeprazine (EGYT Pharmacochemical Works Budapest, Hungary), (-) butaclamol, (+)butaclamol (Research Biochemicals Inc. P.O.Box 181 Wayland), trans(e)-clopenthixol, cis(z)-clopenthixol (Lundbeck CO.A/S. 2500 Kobenhavn-Valby, Denmark) (Figure 1).

Cell cultures. The L5178Y (parent) mouse T cell lymphoma and its transformed subline with mdr (MDR1/A) were grown in McCoy's 5AA medium supplemented with 10 % heat- inactivated horse serum (6).

Fluorescenqe uptake assay. The L5178Y mouse T cell lymphoma cell line was transfected with the pHa *mdr1/A* retrovirus as previously described

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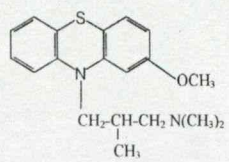
Key Words: Multidrug resistance, blood brain barrier, stereoselectivity, methotrimeprazine, butaclamol, clopenthixol.



maleinat

L-methotrimeprazine
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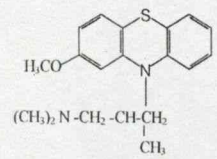

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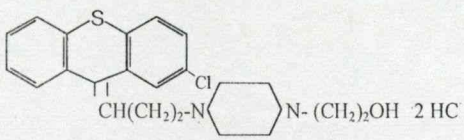
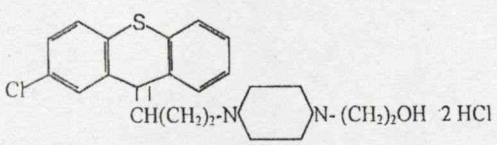


cis - Clopenthixol
4-[3-(2-Chlorothioxanthen-9-ylidene)propyl]-1-piperazineethanol



trans - Clopenthixol
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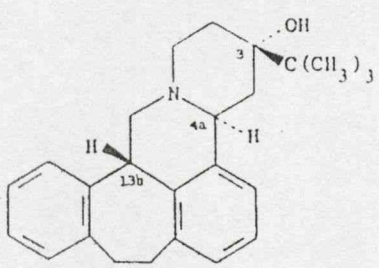

Butaclamol
3-(1,1-Dimethylethyl)-2,3,4,4a,8,9,13b,14-octahydro-1H-benzo[6,7]cyclohepta[1,2,3-de]pyrido-[2,1-a]isoquinolin-3-ol

Figure 1. *The chemical structure of methotrimeprazine, clopenthixol and butaclamol enantiomers and stereoisomers.*

Table I. *Determination of 50 % growth inhibitory dose of various stereoisomers on mdr and parent cells of mouse lymphoma.*

Cell	Compounds	Cytotoxicity ID_{50} (μg/ml)
Parental	(–)butaclamol	0.375
	(+)butaclamol	0.400
	cis-clopenthixol	0.697
	trans-clopenthixol	0.325
	L-methotrimeprazine	0.450
	D-methotrimeprazine	0.375
		ID_{50} (μg/ml)
MDR	(–)butaclamol	0.285
	(+)butaclamol	0.290
	cis-clopenthixol	0.250
	trans-clopenthixol	0.252
	L-methotrimeprazine	0.250
	D-methotrimeprazine	0.325

Table II. *Effect of methotrimeprazine, clopenthixol and butaclamol stereoisomers on the activity of multidrug resistance efflux-pump in mouse lymphoma cells.*

Samples	Conc. μg/ml	FL-1		Fluorescence Activity Ratio
		PAR treated / PAR control	MDR treated / MDR control	
Verapamil	5	0.65	8.43	12.97
L-methotrimeprazine	0.5	0.89	3.20	3.60
	5	0.74	5.62	7.59
D-methotrimeprazine	0.5	0.93	5.58	6.00
	5	0.86	8.32	9.67
Trans(E)-Clopenthixol	0.5	0.93	11.63	12.51
	5	0.66	16.27	24.65
Cis(Z)-Clopenthixol	0.5	0.90	14.47	16.08
	5	0.69	17.29	25.06
(–)Butaclamol	0.5	0.83	6.60	7.95
	5	0.78	17.96	23.03
(+)Butaclamol	0.5	0.93	2.27	2.44
	5	0.84	4.78	5.69

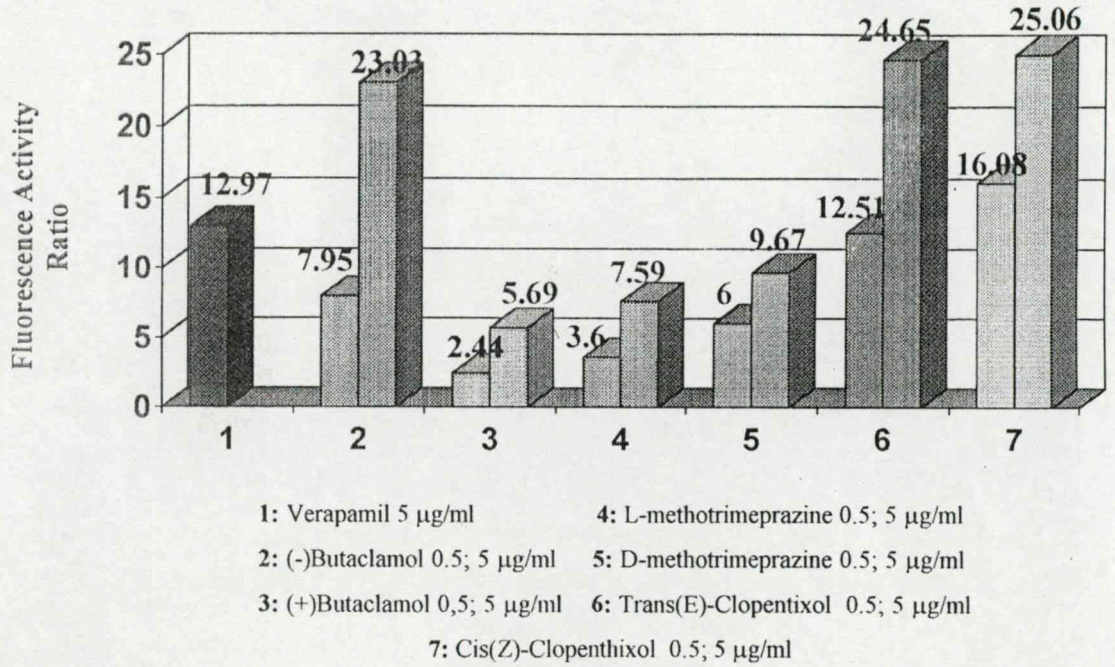

Figure 2. *The effect of butaclamol, methotrimepramine and clopenthixol isomer on the fuction of mdr efflux-pump in mouse lymphoma cells.*

(6). *Mdr1* expressing cells were selected by culturing the transfected cells in 60 ng/ml colchicine. The L5178Y mdr cell line, and the L5178Y parental cell line were grown in McCoy's 5AA medium with 10 % heat inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a concentration of $2x10^6$/ml and resuspended in serum free McCoy's 5AA medium and were distributed into a 0.5 ml aliquots in Eppendorf centrifuge tubes. Test compounds were added from 0.5 to 5 µl of the 1.0 mg/ml stock solutions and the samples were incubated for 10 minutes at room temperature. Then 10 µl of the indicator Rhodamin-123 (5.2 µM final concentration) or doxorubicin was added to the samples and the cells were incubated for further 20 minutes at 37 °C, washed twice and resuspended in 0.5 ml PBS for FACS analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instruments.

Assay for anticellular effects. Greiner 96-well flat-bottom microtrays were used throughout the study. For diluting and distributing cells and reagents, an 8-channel pipette was used. L5178Y parent and mdr tumor cells were resuspended in the growth medium and seeded into the microtrays at appropriate concentrations (4000/0.2 ml/well).

The growth medium was McCoy's 5AA medium containing 10 % horse serum. Then, from 0 to 50 µg/ml of the compounds were added to the wells (5 µl/well). Cell controls and medium controls were set up in each tray. Cell control wells received 0.2 ml/well of growth medium, and medium control wells had no cell suspension but 0.2 ml of growth medium.

The total volume of medium per well at this point was 0.2 ml. The trays were incubated further at 37°C for 3 days after cell seeding in a CO_2 incubator.

At the end of the incubation period, the cells in the wells were stained by 20 µl MTT solution for 4 hours then 100 µl SDS treatment for 24 hours. Inhibition of growth, as well as of the cytotoxic effects of the test samples, were determined and quantitated by measuring the optical density at 545 nm with a Awareness Stat Fax-2100 Technology Inc. vertical beam reader (reference 630 nm).

The average optical density of 2 wells of each dilution, as well as the controls, was calculated and the percentage inhibition was determined according to the formula:

$$100 - \frac{(\text{OD sample} - \text{OD medium control})}{(\text{OD cell control} - \text{OD medium control})} \times 100$$

Results

Before the reversal of multidrug resistance the ID_{50} values of the compounds were determined and it was found that the parent cell line was a little bit less sensitive to the stereoisomer pairs than the multidrug resistant cell line. However, we have to consider that for determination of antiproliferative effect much smaller number of cells (see materials and methods) are treated for much longer time, than for the determination of reversal of mdr efflux-pump (Tablel I).

The effect of three different pairs of stereosimers was studied on the activity of mdr-glycoprotein of the mouse lymphoma cells (Table II). The methotrimeprazine enantiomers *d* and *l* had nearly the same effect by inhibiting the efflux-pump activity of mdr p-glycoprotein in mouse lymphoma cells. The results show that the two enantiomers do not differ significantly in the reversal of multidrug resistance.

The second pair was the butaclamols. An interesting correlation was found between mdr-reversing effect and molecule configuration of the butaclamol stereoisomers. In the experiments the concentration dependent inhibition of stereoisomers on the efflux pump were compared.

Interestingly (-)butaclamol was more effective than the biologically important (+)butaclamol enantiomer. The data indicate that there are no general rules for structure activity relationships in the inhibition of mdr efflux pump activity by tricyclic compounds.

The third stereoisomer pairs are the clopenthixols. The cis- and trans-clopenthixol had similar inhibitory effects on the mdr efflux-pump of mouse lymphoma cells if results were compared to the verapamil as a control.

Since the CNS-inactive members of the tested drugs were as effective on the mdr p-glycoprotein as their active neuroleptic counterpart, we can conclude the lack of stereoselectivity of drug binding in the inhibition of mdr efflux-pump mechanism. However, the remarkable activity of (-)butaclamol is an exception, which is probably based on the higher lipofilicity of the (-)butaclamol than (+) stereoisomer.

Table III. *Comparison of biological effects of some stereoisomeric pairs of methotrimeprazine, butaclamol and clopenthixol.*

Name of compounds	Specificity of pharmacological effect	Experimental dose ID_{50} (nM)*
1	neuroleptic	3.5×10^{-4} M **
Methotrimeprazine		
d		3.5×10^{-4} M **
(+)	neuroleptic	310*
Butaclamol		
(-)		100 000*
cis	neuroleptic	26*
Clopenthixol		
trans		440*

* dopamine stimulated adenylate cyclase activity in rat striatal tissue (13)
** in vitro minimal inhibitory concentration for *E. coli* (26)

Discussion

Chiral anticancer drugs are known since pharmacological differences have been found between stereoisomers. The single isomers of leucovorin, isofosfamide buthionine sulfaximine and verapamil are used in the medical practice with better results than the racemic forms, however, the role of chirality was not reported (71a,71b) in the reversal effect of drugs on the mdr. Despite similar drug targets in the cancer cells, the 50 % inhibitory concentration of various antracyclins, vinca alkaloids, podophillotoxins, topoisomerase inhibitors and antibiotics varied to a great extent in multidrug resistant and sensitive cancer cells (8).

In the mdr cancer cells the efflux-pump mechanism is responsible for treatment failures; therefore, the inhibition of efflux mechanism can result in an effective anticancer chemotherapy. However, we have to consider that normal cells also contain ABC transporters *eg.,* for function of detoxication. To avoid toxic side effects of novel resistance reversal compounds we need drugs with selective inhibition of the mdr efflux in cancer cells. To achieve this effect three classes of known neuroleptic drugs with active and inactive stereoisomers were tested for mdr efflux inhibition and antiproliferative effects on sensitive and multidrug resistant cancer cell cultures and results were compared to verapamil as a classic resistance modifier.

The (-) isomer of verapamil is 10 fold more potent as a calcium antagonist than the (+) isomer, both enanthiomers are equally effective in increasing cellular accumulation of anticancer drugs (9). The enantiomers of phenylalkylamines equally potent in inhibition of drug transport by p-glycoprotein (10). However, the potency of vinca stereo-isomers in both wild type and mdr cells was dependent on the substituents of stereoisomeric form due to modulation of cytotoxicity of vinblastine (11). When the stereoisomers of verapamil and cyclosporines were compared on pgp mediated efflux and NK cell mediated cytolysis verapamils were more potent inhibitors of cytolysis than cyclosporines, on the contrary cyclosporines were more effective on inhibition of pgp mediated rhodamine efflux than verapamil isomers (12).

The CNS - active and inactive - members of butaclamol enantiomers differed in mdr- reversal very much, that means drug binding has some enantioselectivity on the p-glycoprotein. On the contrary, the similar effect of levo- and dextromethotrimeprazine provides idence against the enantioselective inhibition of drug efflux-pump. How can we explain the two different examples of enatiomer effects ? The CNS inactive enantiomers were inhibitory in case of methotrimeprazine and butaclamol as well. Therefore, the mdr reversing effects of CNS inactive members of enantiomers can be exploited in the neoadjuvant chemo-therapy of cancer to increase the effectivity of several cytostatics. The remarkable inhibitory effect of (-)butaclamol can be due to the higher log-P value, the increased lipofilicity.

Surprisingly, the stable stereoisomers of clopenthixol had similar concentration dependent inhibition on the mdr efflux mechanism, for the evidence for the lack of stereospecificity in the drug binding on cancer cells. On the contrary, the normal cells in the CNS (13) and the heart (9), in bacterial plasmid replication (5), inhibition of ATPase or choline-sterase enzymes (5) show that the stereoselectivity and configuration of tricyclic compounds is essential (13).

The cis-clopenthixol inhibited the dopamine stimulated adenyl cyclase 10 fold compared to the trans isomer.

In the case of methotrimeprazine enantiomers, a significant stereoselective effect was observed on dopamine and 5-hydroxi tryptamine receptors, whereas the opiate receptors did not discriminate between the levo- and dextrorotatory isomers (Table III) (14).

The basis of selectivity of stereoisomers may be due to a rigid configuration of the receptor sites. Another reason is the individual members of stereoisomer pairs have different energy levels for binding. Indeed cis-stereoisomer has a more stable configuration with lower energy level than the trans-form of clopenthixol. The differences in the energy levels in the excited state can be even higher (15,16).

Based on the relatively high concentration of drugs used in our experiments, a potential non-specific effect was suggested to develop *via* interaction at various drug receptors (Table I). As an example sigma receptors have high affinity binding sites for several psychotropic drugs. Similar sites are located not only in the CNS but also occur in various periferal tissues. The overexpression of these receptors is found in human tumors (17). Different neuroleptic compounds affecting sigma receptors produced changes in cell morphology. Some neuroleptics lacking sigma affinity such as (+)butaclamol and clozapine had no effect on cellular morphology (18). However, (-)butaclamol exhibited morphological changes (19) resembling apoptosis. The sites are distinguishable by their affinity for stereospecific ligands (19).

The (+)butaclamol has a high affinity binding site on dopamine D_2 receptor (20) while the (-)butaclamol was 300 times less active. Interestingly the (-) enantiomer was more active in the reversal of mdr efflux-pump than the (+)butaclamol, which means that mdr reversal effect was not mediated by D_2-like structures, but probably mediated by sigma receptors. 5-hydroxy tryptamine receptors are suggested to play a role in certain neuroleptic disorders the therapeutic effect of (+)butaclamol and clozapine is localized on 5HT receptors by inhibition some effects of 5HT. However the (-)butaclamol and (-)propranolol were less effective (21).

Considering the role of D_4 receptor or D_4-like structures on the mdr glycoprotein, we can exclude D_4 specific binding on the pgp because dopamine receptor antagonists which showed high affinity to the receptor with a rank order of haloperidol > chlorpromazine > (+)butaclamol > (-)butaclamol. The (+)butaclamol bound to D4 receptors in stereoselective manner showing higher affinity than its respective (-) enantiomer. The (+) enantiomer was found to be nearly 20 fold more effective than (-)butaclamol (22). The lack of dopamine receptor specific stereospecificity of mdr-reversal excludes the involvement of D_2 and D_4-like binding sites on the mdr p-glycoprotein, rather our results refer to the sigma receptor involvement for drug binding responsible for inhibition of drug efflux in the tumor cells.

When mdr cells were exposed to tricyclic stereoisomers with lipophilic characteristics the drug resistance was reversed due to the inhibition of efflux-pump system. However, down-regulation of the mdr gene was also found in some cases (23,24). In control experiments the effect of verapamil stereoisomers had no substantial difference in the potencies of (+) and (-) enantiomers in reversing mdr efflux-pump (10) and in age related clearance (25).

Theoretically the enantioselective mdr-reversal effect of the pharmacologically inactive (-)butaclamol can be exploited in the combination chemotherapy based on the different lipophilicity of the two stereoisomers.

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References

1 Hofsli E and Nissen-Mayer J: Reversal of multidrug resistance by lipophillic drugs. Cancer Res *50:* 3997-4002, 1990.

2 Fisher GA, Brophy NA, Yahanda AM, Adler KA, Lum BL, Bartlett NL, Halsay J and Sikic BI: The modulation multidrug resistance: clinical studies at Stanford. *In:* The Mechanism and New Approach on Drug Resistance of Cancer Cells ed. T. Miyazaki, F. Takaku and K. Sakurada. p.255-266, 1993. Elsevier Science Publishers B.V.

3 Aszalos A: Modulation of multidrug resistance in cancer by immunosuppressive agents. Pathology Oncology Research *1:* 64-70, 1995.

4 Molnár J, Szabó D, Csuri K and Motohashi N: Cytotoxicity and calculation of half-mustard type phenothiazines. Anticancer Research (1998) in preparation.

5 Molnár J, Gálfi M, Lózsa A and Nakamura MJ: Inhibition of bacterial plasmid replication by stereoselective binding by tricyclic psychopharmacons. Research Comm. in Chem. Pathol. and Pharmacology *43/2:* 235-249, 1984.

6 Cornwell MM, Pastan I, Gottesmann MM: Certain Calcium channel blockers bind specifically to multidrug resistant KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J. Biological Chemistry *262:* 2166-2170, 1987.

7/aWainer IW, Granvil CP: Stereoselective separations of chiral anticancer drugs and their application to pharmacodynamic and pharmacokinetic studies. Ther Drug Monit Dec, 15 (6): 570-5, 1993.

7/bWainer IW: Stereoisomers in clinical oncology: Why it is important to know what the right and left hands are doing. Ann. Oncol *4 Suppl 2:* 7-13, 1993.

8 Hiroshi Isobe, Awtar Krishan, Larry Wellham, Yoshisaku Kawakami: Drug resitance and p-glycoprotein expression, DNA topoisomerase II activity and glutatione content in malignant mesothelioma cell lines establised after *in vivo* chemotherapy. *In:* The Mechanism and New Approach on Drug Resistance of cancer cells . T. Miyazaki, F. Takaka and K. Sakurada, Elsevier Science Publishers p. 103- 114, 1993.

9 Gruber A, Peterson C, Reizenstein P: D-verapamil and L-verapamil are equally effective in increasing vincristine accumulation in leukemic cells *in vitro.* International Journal of Cancer *41:* 224-226, 1988.

10 Hoolt V, Kouba M, Dietel M, Vogt G: Stereoisomers of calcium antagonists which differ markedly in their potencies as calcium blockers are equally effective in modulating drug transport by P-glycoprotein. Biochem. Pharmacol. Jun 23; *43 (12):* 2601-8 , 1992.

11 Bormann LS, Bornmann WG, Kuehne ME: Modulation of drug cytotoxicity by stereoisomeric series of C-20 vinblastine congmers that lack antimicrotubule activity. Cancer- Chemother-Pharmacol *31/5:* 343-9, 1993.

12 Klimecki WT, Taylor CW, Dalton WS: Inhibition of cell-mediated cytolysis and P- glycoprotein function in natural killer cells by verapamil isomers and cyclosporine A analogs. J Clin Immunol May *15(3):* 152-8, 1993.

13 Hyttel J: Effects of neuroleptics on ^{3}H-haloperidol and ^{3}H-cis-Z-flupenthixol binding and on adenylate cyclase activity *in vitro.* Life Sciences *23:* 551-556, 1978.

14 Robert TA, Hagardorn AW and Daigneault EA: Differential

stereoselectivity of metotrimiprasine enantiomers for selected central nervous system receptor types. Molec Pharmacol *21:* 315-319, 1982.

15 Cumdell RB and Gilbert A: Cis-trans isomerism, *In:* Nelson T. ed., Photochemistry Studies in Modern Chemistry. Academic Press, New York, N.Y. P. 168, 1970.

16 Motohashi N, Kurihara T, Kawase M, Hever A, Tanaka M, Szabo D, Nacsa J, Yamanaka W, Kerim A, Molnar J: Drug resistance reversal, antimutagenicity and antiretroviral effect of phthalimido-and chlorethyl-phenothiazines. Anticancer Res (in press).

17 Bean WT, Thomas GE, Mamone JY, Homan SM, Levy BK, Johanson FE, Coscia CJ: Overexpression of sigma receptors in non-neural human tumors. Cancer Res *51:* 6558-62, 1991.

18 Vilner BJ, de Costa BR, Bowen WD: Cytotoxic effects of sigma ligands: sigma receptor-mediated alterations in cellular morphology and viability. J-of Neuroscience *15/1:* 117-134, 1995.

19 Hellewell SB, Bowen WD: A sigma-like binding site rat pheochromocytoma PC12 cells. Brain Res *527:* 244-253, 1990.

20 Largent BL, Wilestrom H, Gandlach AL, Snader SH: Structural determinants of sigma receptor affinity. J Mol Pharmacol *32:* 772-784.

21 Plassat JL, Amlaiky N and Hen R: Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Molecular Pharmacology *44:* 229-236, 1993.

22 Patel S, Marwood R, Emms F, Marston D, Leeson PD, Curtis NR, Kulagowski JJ and Freedman SB: Identification and Parmacological characterisation of ^{125}I-L-750. 667, Molecular Pharmacology *50:* 1658-1664, 1996.

23 Molnár J, Szabó D, Mándi Y, I Mucsi, Fischer J, Varga A, S König, Motohashi N: Multidrug resistance reversal in mouse lymphoma cells by heterocyclic compounds. Anticancer Res *18:* 3033-3038, 1998.

24 Hevér A, Santelli-Rouvier C, Brouant P, Khyari S, Molnár J, Barra Y, Barbe J: Effect of new thioacridine derivatives on p-gp function and on mdrl gene expression. Anticancer Res *18:* 3053-3058, 1998.

25 Schwartz JB, Capili H, Wainer IW: Verapamil stereoisomers during racemic verapamil administration: effects of aging and comparisons to administration of individual stereoisomers. Clin. Pharmacol. Ther. Oct; *56(4):* 368-76, 1994.

26 Molnár J, Gálfi M, Lózsa A, Nakamaru MJ: Inhibition of bacterial plasmid replication by stereoselective binding by tricyclic psychopharmacons. Research Communications in Chemical Pathology and Pharmacology *43:* 235-249, 1984.

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III.

Multidrug Resistance Reversal in Mouse Lymphoma Cells by Heterocyclic Compounds

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Abstract. *Due to the close homology between bacterial and tumor cell transporter proteins, some antiplasmid and anticancer compounds were tested for their ability to reserve the multidrug resistance (mdr) of lymphoma cells. Some known anticancer medicines such as platidiam, novantron, fluorouracil, bleomycin and methotrexate were ineffective/while vinca alkaloids exerted a strong reversal effect on the mdr of lymphoma cells. The structurally related reserpine and yohimbine do not affect the activity of efflux pump. Some selected antitumor phenothiazines and benzo[a]phenothiazines, including trifluoperazine inhibit the P-glycoprotein (pgp) function. This fact is independent from the antiproliferative- or differentiation inducing effects. Since the polylactosamine specific tomato lectin prevents the action of the chemosensitizers tested, it is supposed that the site of action of phenothiazines can be at the 1st loop in the transmembrane glycoprotein. The efflux pump activity of the pgp in brain capillary endothel which is responsible for blood brain barrier (BBB) was also inhibited by some phenothiazines. However, the tomato lectin sensitivity of pgp was different in mouse lymphoma and human brain capillary endothelial cells. The mdr-gene expression of the mouse lymphoma cells (which were transfected with the human mdr-1 gene) could be reduced by phenothiazines such as promethazine and trifluoperazine, when the cells were cultured in the presence of 0.5 μg/mL phenothiazines. Further synergism was found between two resistance modifiers i.e. verapamil and trifluoperazine on the inhibition of mdr-glycoprotein.*

Cancer is the 3rd most important cause of death worldwide, and lung cancer of questionable origin is the most frequent of these malignancies. However, WHO has estimated that over 1.5 million of the total of ten million new cancer cases per year are associated with infections such as *Helicobacter pylori*, human papillomaviruses and hepatitis viruses, not to mention others, such as HTLV infections. Approximately 40% of cancer patients can be treated by surgery and the great majority of patients need chemotherapy. The effectiveness chemotherapy is limited by the emergence of drug resistance, especially multidrug resistance (*mdr*).

mdr is associated with increased expression of 170 kD *mdr*-1 P-lycoprotein (pgp) (1), which extrudes anticancer drugs from the cells. Apart from *mdr* (pgp), other resistance mechanisms exist, e.g., the *mdr*-associated protein (MRP) (2), which is mediated by a-190 kD membrane glycoprotein (3), and the lung resistance protein (LRP) which a 110 kD protein involved in nuclear cytoplasmic drug transport (4). Enhanced glutathione-S-transferase (5) vesicular drug binding and compartmentization of drugs, *e.g.*, mitoxantrone (6) and DNA topoisomerase mutation-related resistance have been also observed in tumor cells. The wide spread use of anticancer drugs underlines the importance of the elucidating the various mechanisms of resistance, especially the characterization of different *mdr* phenotypes. New compounds could be introduced to overcome multidrug resistance by blocking the enhanced drug efflux with the help of new resistance modifiers (7), by introducing more specific antitumor drugs against various cancer cell lines (8), or molecules targeting phosphorylation, protein kinase, differentiation and telomerase (9).

The new information-intensive approach to cancer chemotherapy (8, 10, 11, 12) has also been used to search for candidate anticancer drugs that are specific for certain types of cancer (13). Our recent studies have focused on the most important *mdr*-glycoprotein mediated drug resistance which can be considered the most widely distributed form of resistance in bacteria (14, 15, 16, 17, 18), fungi (19), helmints (20), and cancer cells (14, 21, 22). Considering the close homology between these ABC transporter proteins, a systematic study was initiated to develop new *mdr* reversing compounds from anticancer- and antiplasmid- and

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Key Words: Multidrug resistance, blood brain barrier, phenothiazines, tomato lectin.

Table I. *The effect of some anticancer drugs and resistance modifiers on rhodamine-123 accumulation by mdr mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
verapamil	8.0	2.29
platidiam	2.0	0.53
	20.0	0.60
novantron	2.0	0.67
	20.0	0.70
fluorouracil	2.0	0.47
	20.0	0.73
bleomycin	0.5	0.88
	5.0	0.80
methotrexate	5.0	1.00

Table II. *The effect of some vinca alkaloids and structurally related compounds on R123 accumulation by mdr mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
thaliblastine	2.0	20.38
	20.0	13.50
vinblastine	2.0	22.33
	20.0	23.89
vincristine	2.0	16.05
	20.0	34.51
reserpine	2.0	0.85
	20.0	1.43
yohimbine	2.0	0.49
	20.0	0.68

immunomodulatory agents. The possible synergistic action with anticancer drugs also will be discussed.

Materials and Methods

Chemicals. Chlorpromazine (CPZ), 6,9-dioxochlorpromazine, 7,8- dioxo-7-semicarbazone-chlorpromazine, 5-oxo-5*H*-6-hydroxybenzo[*a*]phenothiazine and 6-methyl-5- oxobenzo[a]phenothiazine were synthetized as described previously (23). 7,8-Dihydroxychlorpromazine and 7,8-dioxochlorpromazine were synthetized as described previously (24). Verapamil and promethazine were obtained EGIS Works, Hungarian Pharmaceutical Company, Budapest. Platidiam (Lachema Brno), novantron, fluorouracil (La Roche Ltd Basel), bleomycin, methotrexate (Lachema-Brno), trifluoroperazine, thaliblastine (TBL), vincristine (Richter-Gedeon), vinblastin (Richter-Gedeon), reserpine, yohimbine, cyclosporin A (Sandimmune, Cs-A) and cyclosporin D (PSC-833, Cs-D).

Cell culture. For the cytotoxicity assay, HEp-2 cells were cultivated in RPMI supplemented with 10% fetal calf serum. The L5178Y (parent) mouse T cell lymphoma and its transformed subline with *mdr* (*MDR*1/A) were grown in McCoys 5AA medium supplemented with 10% heat-inactivated horse serum (25). HEp-2 cells for immunofluorescence studies were grown on a glass coverslip in Petri dishes in Eagle MEM supplemented with 5% new born calf serum.

Fluorescence uptake assay. The L5178Y mouse T cell lymphoma cell line was infected with the PHA *mdr*-1/A retrovirus as previously described (25). *Mdr*-1 expressing cells were selected by culturing the infected cells in 60 ng/mL colchicine. The L5178Y *mdr* cell and L5178Y parental cell line were grown in McCoys 5AA medium with 10% heat inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum free McCoys 5AA medium and the cells were distributed into a 0.5 mL aliquot in Eppendorf centrifuge tubes. Test compounds were added to 1.0-10 μL of the 1.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Then 10 μL of the indicator rhodamin-123 (5.2 μM final concentration) was added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instruments.

The effect of lectins on P-glycoprotein. This was studied by fluorescence uptake assays. Tomato lectin was added to *mdr* cell line or brain capillary endothelial cells at different concentrations in the presence of 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$.

The inhibition of mdr gene expression. The par and *mdr*-1 cell lines were incubated in the presence of 0.5-2.0 μg/mL trifluoperazine and promethazine for 48 hours at 37°C in 5% CO_2 as usual. For isolation of the total cellular RNA, the cells were washed in ice cold sodium chloride, centrifuged and RNA was prepared from drug treated and control cells with miniprep-RNA protocol cDNA was prepared of the 1.0 μg isolated RNA by reverse transcriptase (26). The reverse transcriptase (RT) assay was run at 42°C for 60 min followed by denaturation at 95°C for 5 min and a cooling step at 5°C for 5 min. The amplification was performed in polymerase chain reaction (PCR) at 94°C for 5 min and continued for 37 cycles of melting 94°C for 30 seconds and annealing extending with Taq polimerase 62°C 50 seconds followed by a final step at 72°C 60 seconds. The PCR products were separated in 2.0% agarose in the presence of ethidium and semiquantitated by densitometry Wayne-Rasband, NIMH, Bethesda, U.S.A. The trifluoperazine and promethazine apparently inhibited the synthesig of *mdr*-1 RNA by the inhibition of gene expression primers for PCR were *mdr*-1 and ß-actin.

Results

First of all, some well known anticancer drugs were tested for multidrug resistance (*mdr*) reversing activity. Platidiam, novantron, fluorouracil, bleomycin and methotrexate did not affect the efflux pump activity (Table I). However, some vinca alkaloids, *i.e.*, vinblastine, vincristine and thaliblastin enhanced the rhodamine 123 accumulation in the *mdr* cells. The structurally similar reserpine had very slight effect and yohimbine was ineffective (Table II). Some ring substituted chlorpromazine derivatives had moderate effect or were ineffective. Benzo[a]phenothiazines were effective at relatively high concentrations from 2.0 to 20 μg/ml, and in this study 5-oxo-5H-benzol[a]phenothiazine was the most active compound (Table III). Some phenothiazine compounds were more effective on the drug efflux of human brain capillary endothelial cells than on *mdr* cells (Table IV), but the inhibition was low. While the Cs-A and Cs-D were much more inhibitory on the *pgp* function of mouse lymphoma cells

Table III. *The effect of some phenothiazines and benzo[a]phenothiazines on R123 accumulation by mdr mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
7,8-dioxochlorpromazine	2.0	0 .89
	20.0	0.70
6,9-dihydroxychlorpromazine	2.0	0.82
	20.0	0.79
6,9-dioxochlorpromazine	2.0	0-79
	20.0	2.38
5-oxo-5*H*-benzo[*a*]phenothiazine	2.0	0.85
	20.0	1.40
6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine	2.0	1.20
	20.0	1.00
6-methyl-5-oxo-benzo[*a*]phenothiazine	2.0	3.44
	20.0	2.92
verapamil	8	3.81

Table IV. *The effect of substituted phenothiazines on R123 accumulation by human brain capillary endothelial cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
capillary endothel (control)	–	–
verapamil	8	0.81
6,9-dihydroxychlorpromazine	2	1.06
	20	1.03
7,8-dioxochlorpromazine	2	1.11
	20	0.85
6,9-dioxochlorpromazine	2	1.05
	20	0.80
5-oxo-5*H*-benzo[*a*]phenothiazine	2	1.10
	20	0.69
FC-1884	2	1.18
	20	1.11

than the previously tested phenenothiazines. Cs-D had a higher activity than Cs-A (Table V), which gives an opportunity to improve the *mdr* reversal effect by further changes in the chemical structure of the polypeptide (Figure 1).

The *mdr* reversal effect of promethazine was decreased by tomato lectin treatment (Table VI). The binding of promethazine or trifluoperazine to the *pgp* of the tumor cells and to brain capillary endothelia was determined by the interaction of tomato lectin with polylactosamine moiety of the first loop of *pgp*, meaning that the 1st loop has a key role in the biological effect of *pgp*. The tomato lectin itself was not able to affect the efflux pump activity in the tumor cells, but the inhibition of promethazine (or trifluoperazine) was reduced. An opposite effect was observed on human brain capillary endothelial cells.

The tomato lectin reduced the inhibitory effect of promethazine (or trifluoperazine); however, the lectin was able to inhibit the efflux pump activity of the *pgp* of the brain capillary endothelial cells (Table VI) showing that the localization (or accessibility) of the *pgp* is not exactly the same in the two cell lines.

In the experiments, the presence of *pgp* in drug treated brain capillary endothelial and lymphoma cells were examined in trypsinized and acetone fixed cells by using two monoclonal antibodies. One of the *mab 4E 3.16* (SIGNET Laboratories, Inc, Mass, U.S.A) requires the epitope of *mdr*-1 on the exterior surface of the cell membrane. The 2nd, C219 (SIGNET Laboratories) recognizes an internal highly conserved sequence found in both *mdr*-3 and *mdr*-1 isoforms of *pgp*, and was studied under the fluorescent microscope. The external and internal fragments of *pgp* were identified in both cell lines. Trypsin treatment (0.25% trypsin for 5 min) markedly decreased the specific expression binding of mab for external fragment of *pgp* on the surface of brain capillary

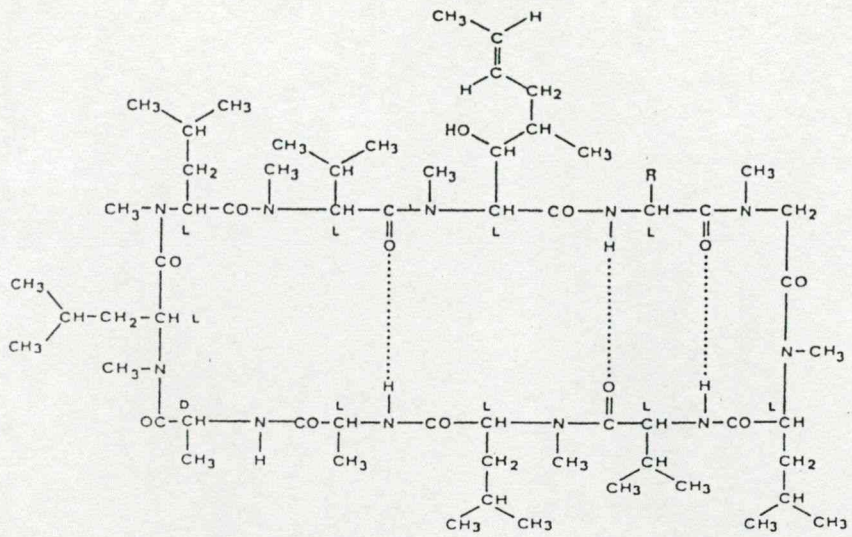
Table V. *The effect of cyclosporins on R123 accumulation by mdr mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
cyclosporin-A	2	12.72
(Sandimmune)	4	24.87
	8	50.04
	10	51.00
cyclosporin-D	2	99.29
(PSC-833)	4	100.47
	8	102.78
	10	112.57

endothelial or lymphoma cells. The binding of *mab* to the internal compartments of *pgp* was not changed. It is possible that more than one mechanism is involved in the action of phenothiazines and tomato lectin on the efflux pump of tumor cells.

At any rate, phenothiazine and trifluoperazine reduced the gene expression in the tumor cells, when the cells were cultured in the presence of low concentrations of the drugs for 48 hours (Figure 2). The results show that at least two independent biological processes are responsible for reversal of *mdr* of tumor cells such as the reduced activity of the efflux pump and the down regulation of *mdr* gene.

Since the *pgp* responsible for the *mdr* of the tumor cells also has a physiological function, (*i.e.* maintain the blood-brain barrier (BBB)), it was reasonable to search for specific *mdr* reversing compounds and selective antitumor drugs (Table VII). For improving the selectivity of anticancer compounds, some phenothiazines were synthetized with alkylurea and "half- mustard type" phenothiazines and tested on sixty cancer cell lines (27). There were some differences



Cyclosporin A: R=-CH_2CH_3
Cyclosporin D: R=-$CH(CH_3)_2$

Figure 1. *Chemical structure of cyclosporin A (Sandimmune) and cyclosporin D (PSC 833).*

between the growth inhibitory potencies of the compounds on the various cancer cell lines showing that specificity of anticancer agents can be increased by substitutions (12,13,27).

However, the *mdr* reversing effect-of some representatives of the newly synthesized "half-mustard type" phenothiazines was nearly the same (8, 11, 13).

Furthermore, the specificity of *mdr* reversal effect was analysed by using stereoisomers of some active compounds (28). As a result of these experiments the selectivity of the inhibition, on the *mdr* efflux pump was improved (29). Putting the two findings together, we realized that their is an opportunity to improve the perspectives of cancer chemotherapy by combination chemotherapy (Table VIII). The combination of trifluoperazine and verapamil was found to be the most effective in this system.

Discussion

The large number of cancer patients and the emerging *mdr* of cancer cells leads to the ineffectiveness of chemotherapy. The majority of anticancer chemotherapy medicines do not inhibit *mdr* but can induce the expression of the *mdr* gene. Therefore, physicians have to change the strategy of chemotherapy. One way is to improve the effectiveness of chemotherapy is to inhibit the resistance mechanism, the second is to prevent the induction of *mdr* and the third is to develop new types of anticancer drugs.

Since *mdr pgp* is involved in the acquired *mdr* of tumor cells, the selective killing of cancer cells, and expression of *pgp*, could be improved by the simultaneous administration of anticancer drugs and resistance modifiers in combination

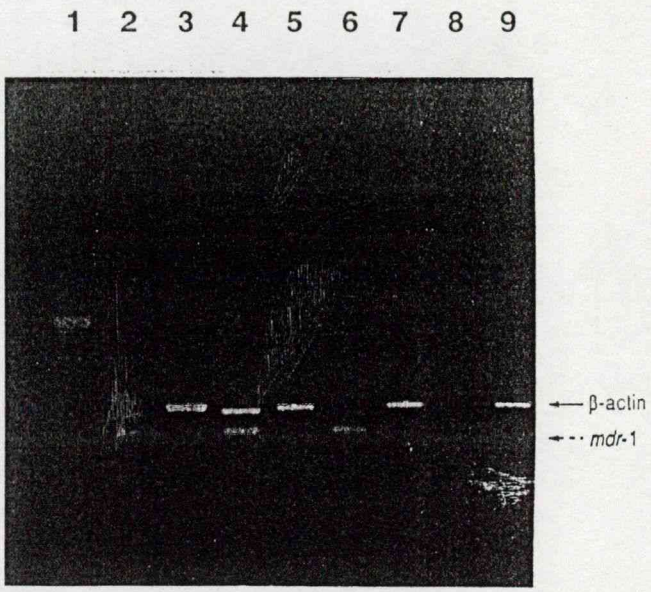


Figure 2. *Reverse transcriptase-polymerase chain reaction (RT- PCR) analysis of mdr-1 messenger RNA expression in the presence of trifluoperazine (TFP).* Lane 1. *DNA molecular weight markers (Boehringer, Mannheim, Germany).* Lane 2. *mdr cells grown in the presence of 0.5 μg TFP (mdr without β-actin band).* Lane 3. *mdr cells grown in the presence of 0.5 μg TFP (mdr-1 and β-actin bands).* Lane 4. *mdr cells untreated (mdr-1 and β-actin bands).* Lane 5. *mdr cells grown in the presence of 2.0 μg promethazine (β-actin primer without mdr-1 primer).* Lane 6. *mdr control primer (without β-actin primer).* Lane 7. *control parent cells (with β-actin primer).* Lane 8. *negative control, parent cells only (mdr primer).* Lane 9. *mdr-1 cells grown in the presence of 2.0 μg promethazine (β-actin and mdr- 1 bands).*

Table VII. *Theoretical applications for general efflux-pump inhibitors and organ-specific efflux-pump inhibitors in combination with chemotherapeutics.*

Type		Specificity of	
	mdr-reversing compound's effect	Anticancer drug accumulation	Expected results of combination
A	GENERAL efflux pump inhibitors: quinidine cyclosporin	SPECIFIC for: tissue or organ	synergy
		GENERAL for: uniform distribution in the body	toxic or general immuno-suppresion
B	SPECIFIC for tissue ororgan: verapamil (cardial) trifluoperazine (CNS) thiazinamium (lung)	SPECIFIC for: affinity to CNS kidney, liver etc	strong synergy
		GENERAL for cytotoxic uniform distribution in the body	synergy or organ-specific toxicity

Table VIII. *The effect of drug combinations on the mdr reversal in mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
verapamil	–	–
melipramin	5	7.35
trifluoperazine	5	23.40
amitriptilin	1	3.76
	5	5.66
verapamil + melipramin	2.5 + 5	1.54
verapamil + trifluoperazine	2.5 + 2.5	15.21
verapamil + amitriptilin	2.5 + 0.5	3.69
trifluoperazine + amitriptilin	2.5 + 0.5	2.44

Table VI. *Effect of tomato lectin on the mdr of mouse lymphoma and human brain capillary endothelial cells in the presence of promethazine.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
mouse lymphoma (control)	–	–
promethazine	5	3.80
tomato lectin	50	0 90
tomato lectin + promethazine	50 + 5	2.56
human brain capillary endothel (control)	–	–
promethazine	5	0.71
tomato lectin	50	1.17
tomato lectin + promethazine	50 + 5	0.82

chemotherapy. However, side effects of resistance modifiers on normal cells, expressing the *pgp*, should also be considered because of the organospecific toxicity depends on the amount of *pgp* in the various tissues. *Mdr pgp* is also present in various normal cells (like capillary endothel in brain, colon, kidney and liver cells) and is associated with physiological functions. The *pgp*, expressed in these tissues, is responsible for the transport of toxic compounds into the cerebrospinal fluid (CSF), bile or urine, etc. Consequently, the reduced blood-biliary, blood-urine or BBB blood brain barrier can increase the organotoxicity of antitumor drugs. Therefore, cancer or organospecific efflux inhibitors can be used to increase the cytostatic drug concentration in malignant cells to avoid side effects in other essential organs. For example, if there were drugs which affect the BBB selectively then they could be used for a different purpose. Such drugs would be able to increase the concentration of cytostatics in the tumor cells localized in the brain.

Mdr reversing drugs could increase the concentration of CNS targeted medicines of any kind simultaneously.

On the other hand, even a general chemosensitizer can enhance the uptake of the organospecific medicines, that are subjects of *mdr* efflux pump. Cancer or organ specific efflux inhibitors theoretically can reduce the general toxic effects of cytostatic compounds, due to the increased accumulation of cytostatics in the cancer cells even at reduced administration doses.

In our experiments, the physiological function of one expressed in brain capillary endothelial and lymphoma cells from mice was compared in the transport of R-123 as an indicator in the presence of CNS active medicines.

The results show that there are some differences in the lectin sensitivities of-the two cell lines. We suppose that the difference could be the consequence of different localization of the two efflux proteins in the membrane lattice.

If there are differences in drug sensitivity of different cells, one can improve the activity or selectivity of a given compound with rational drug design, *e.g.*, the modification of phenothiazine ureas and "half-mustard type" phenothiazines (8) or by introducing changes into Cs-A resulting in a higher mdr reversing effect with Cs-D than Cs-A based on the modified structure (28). When many structurally related phenothiazines were tested for their antiproliferative effects on 60 different cancer cell lines, a wide range of biological activity was observed on a large variety of cancer cell lines (12,13,27, 29). Based on the measured antiproliferative effects, some correlations were found between the sensitivity on different types of cancer cell lines and the structures of substituted phenothiazines (11, 13, 30), meaning that certain types of cancer cell lines are more sensitive than others.

The selectivity of *mdr* reversing compounds can be improved by combination with other chemosensitizers *e.g.*, trifluoperazine plus verapamil, or by exploiting the stereoselectivity (28, 29). In this case, the inactive member of a pair of stereoisomers should be considered to inhibit the *mdr* pgp.

Since promethazine and trifluoperazine are able to downregulate *mdr* gene expression, the pretreatment of cancer cells with these (or less toxic) compounds make the cells more sensitive to anticancer drugs by preventing the induction of *mdr*.

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References

1 Endicott JA and Ling V: The biochemistry of P-glycoprotein-mediated multidrug resistance. Ann Rev Biochem *58:* 137-171, 1989.

2 Hasegawa S, Abe T, Naito S, Kotoh S, Kumazawa J, Hipfner DR, Deeley RG, Cole SPC and Kuwano M: Expression of multidrug resistance-associated protein (MRP), *MDR*1 and DNA topoisomerase II in human multidrug-resistant bladder cancer cell lines. Brit J Cancer *71:* 907-913, 1995.

3 Zaman GJR, Flens MJ, van Leusden M-R, de Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ and Borst P: The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA *91:* 8822-8826, 1994.

4 Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen THM, van Kalken CK, Slovak ML, de Vries EGE, van der Valk P, Meijer CJLM and Pinedo HM: Overepression of a *Mdr* 110,000 versicular protein in non-P-glycoprotein- mediated multidrug resistance. Cancer Res *53:* 1475-1479, 1 993.

5 Whelan RDH, Hosking LK, Townsend AJ, Cowan KH and Hill BT: Differential increases in glutathione S-transferase activities in a range of multidrug-resistant human tumor cell lines. Cancer Commun *1:* 359-365, 1989.

6 Kellner U, Hutchinson L, Seidel A, Lage H, Danks MK, Dietel M and Kaufmann SH: Decreased drug accumulation in a mitoxantrone-resistant gastric carcinoma cell line in the absence of P-glycoprotein. Int J Cancer *71:* 817-824, 1997.

7 Molnar J, Hever A, Fakla I, Fischer J, Ocsovszki I and Aszalos A: Inhibition of the transport function of membrane proteins by some substituted phenothiazines in E. coli and multidrug resistant tumor cells. Anticancer Res *17:* 481-486, 1997.

8 Motohahsi N, Kawase M, Kurihara T, Hever A, Nagy Sz, Ocsovszki I, Tanaka M and Molnar J: Synthesis and antitumor activity of 1-(2-chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkylureas as potential anticancer agents. Anticancer Res *16:* 2525-2532, 1996.

9 Aszalos A and Eckhardt S: Molecular events as targets of anticancer drug therapy. Pathology Oncology Res *3* (No2): 147-158, 1 997.

10 Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace AJ, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE and Paull KD: An information- intensive approach to the molecular pharmacology of cancer. Science *275:* 343-349, 1997.

11 Motohashi N, Kurihara T, Kawase M, Hever A, Tanaka M, Szabo D, Nacsa J, Yamanaka W, Kerim A and Molnar J: Drug resistance reversal, anti-mutagenicity and antiretroviral effect of phthalimido- and chloroethyl-phenothiazines. Anticancel Res *17:* 3537-3543, 1997.

12 Wuonola MA, Palfreyman MG, Motohashi N, Kawase M, Gabay S, Nacsa J and Molnar J: The primary *in vitro* antitumor screening of "half-mustard type" phenothiazines. Anticancer Res *17:* 3409-3423, 1997.

13 Molnar J, Szabo D, Csuri K, Kurihara T and Motohashi N: Possible correlation between chemical structure and tumor type specificity of phenothiazine derivatives or half- mustard type of phenothiazines. Anticancer Res *18:* in preparation, 1998.

14 Gottesman MM and Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. Ann Rev Biochem *62:* 385-427, 1993.

15 Andreana A, Gollapudi S and Gupta S: Salmonella typhimurium induces expression of P glycoprotein (multidrug resistance 1 gene product) in a promonocytic cell line chronically infected with human immunodeficiency virus type 1. J Infect Dis *169:* 760-765, 1994.

16 Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat S, Yamagishi J, Li X and Nishino T: Overexpression of the mexC-mexD-oprJ efflux operon in nfxB-type multidrug- resistant strains of Pseudomonas aeruginosa. Mol Microbiol *21:* 713-724, 1996.

17 Matthysse AG, Yarnall HA and Young N: Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. J Bacteriol *178:* 5302-5308, 1 996.

18 Gerlach JH, Endicott JA, Juranka PF, Henderson G, Sarangi F, Deuchars KL and Ling V: Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature *324:* 485-489, 1986.

19 Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M and Bille J: Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolated from AIDS patients involve specific multidrug transporters. Antimicro Agents Chemother *39:* 2378-2386, 1995.

20 Broeks A, Gerrard B, Allikmets R, Dean M and Plasterk RHA: Homologues of the human multidrug resistance genes MRP and *MDR* contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J *15:* 6132-6143, 1 996.

21 Hegewisch-Becker S, Fliegener M, Tsuruo T, Zeller W and Hossfeld DK: Multidrug resistance (*mdr* 1) in adult acute leukemia at diagnosis: correlation with response to induction chemotherapy. Onkologie *16:* 264-269, 1993.

22 Ichikawa M, Yoshimura A, Furukawa T, Sumizawa T, Nakazima Y and Akiyama S: Glycosylatin of P-glycoprotein in a multidrug-resistant KB cell line, and in the human tissues. Biochim Biophy Acta *1073:* 309-315, 1991.

23 Motohashi N: Test for antitumor activities of phenothiazines and phenoxazines. (in Japanese). Yakugaku Zasshi *103:* 364-371, 1 983.

24 Hewlett I, Lee S, Molnar J, Foldeak S, Pine PS, Weaver JL and Aszalos A: Inhibition of HIV infection of H9 cells by chlorpromazine derivatives. J Acquired Immun Deficiency Syndrom Human Retrovirol *15:* 16-20, 1997.

25 Cornwell MM, Pastan I and Gottesman MM: Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem *262:* 2166-2170, 1 987.

26 Stein U, Walther W and Shoemaker H: Reversal of multidrug resistance by transduction of cytokine genes into human colon carcinoma cells. J Nat Cancer Inst *88:* 1383-1392, 1 996.

27 (a) Palfreyman MG, Wuonola M, Motohashi N, Kawase M, Gabay S, Gupta RR and Molnar J: The primary *in vitro* anticancer activity of "half-mustard type" phenothiazines in NCI's revised anticancer screening paradigm. Anticancer Res *17:* in press, 1997. (b) Palfreyman MG, Wuonola M, Motohashi N, Kawase M, Gabay S and Molnar J: Phenothiazine compounds, compositions and methods of using the same. U.S. Patent in press, 1998.

28 Keller RP, Altermatt HJ, Donatsch P, Zihlmann H, Laissue JA and Hiestand PC: Pharmacologic interactions between the resistance-modifying cyclosporine SDZ PSC 833 and etoposide (VP 16-213) enhance *in vivo* cytostatic activity and toxicity. Int J Cancer *51:* 433-438, 1992.

29 Szabó D and Molnár J: The role stereoselectivity of chemosensitizers in the reversal of multidrug-resistance of mouse lymphoma. In preparation for Anticancer Res *18:* 1998.

30 Molnár J, Mándi Y, Hever A, Ocsovszky I, Földeák S, Schneider J, Emody L and Aszalos A: Reversal of drug resistance-modifying cyclosporine SDZ PSC 833 and etoposide (VP 16-213) enhance *in vivo* cytostatic activity and toxicity. Int J Cancer *51:* 433-438, 1992.

29 Szabó D and Molnár J: The role stereoselectivity of chemosensitizers in the reversal of multidrug-resistance of mouse lymphoma. In preparation for Anticancer Res *18:* 1998.

30 Molnár J, Mándi Y, Hevér A, Ocsovszky I, Földeák S, Schneider J, Emody L and Aszalos A: Reversal of drug resistance in bacteria and tumor cells by some substituted phenothiazines. Anticancer Res *15:* 1648, 1995.

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IV.

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6,12-Dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones: Synthesis and *mdr* Reversal in Tumor Cells

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Abstract. *Six 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones and three coumarins were systematically investigated for reversal of multidrug resistance of bacteria and cancer cells in model experiments. 7-Methylcoumarin was able to eliminate the E. coli plasmid significantly; however, the other derivatives were ineffective. Four of 6,12-dihydro-1-benzopyrano[3,4-b][1,4] benzothiazin-6-ones had a moderate effect on the multidrug resistance efflux pump of mouse lymphoma cells in vitro. Despite of the similarity of resistance mechanisms of bacteria and tumor cells, the reversal of drug resistance in bacteria and in cancer cells is not uniform because the structure- activity requirements are apparently different.*

In cancer therapy, the development of resistance, including multidrug resistance for traditionally used medicines, is now a major problem. Patients need new type of medicines or special drugs to reduce the drug resistance. Therefore, some benzo[*a*]phenothiazines were planned for synthesis in our laboratories. As shown earlier, benzo[*a*]phenothiazines have antitumor activity against some tumor cells (1), induce significant apoptosis on human myelogenous leukemic cell lines such as ML-1, U-937 and THP-1 (2), and affect the element content of tobacco tissue culture and hormone requirement (3). 6,12-Dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones are structurally very similar to the structures of benzo[*a*]phenothiazines (Figure 1). Some 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones showed an effect on the multidrug resistance efflux pump of mouse lymphoma cells *in vitro* (4). The coumarins which are included in these compounds have also shown antitumor activity by the inhibition of HIV integrase (5). Based on our estimated structure-activity relationship, it was expected that 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones may have some antitumor activity and may cause reversal on resistant tumor cells. The purpose of this paper is to show the *mdr* reversal activity of seven 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones on tumor cells with multi-drug resistance.

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Key Words: 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones, *mdr* reversal on tumor cells.

Materials and Methods

Melting points were determined in open glass capillaries in a paraffin bath and are uncorrected. ^{1}H-NMR spectra were performed on a JEOL JNM-GSX 500 (500 MHz) spectrometer using TMS as internal standard (δ=0). Aromatic protons are represented as ArH. Infrared (IR) spectra were recorded on a JASCO IR810 spectrometer. Mass spectra (MS) were recorded on a JEOL-JMS-DX300 spectrometer with direct inlet system at 70eV. TLC (Thin layer chromatography) was performed on a Merck Kieselgel 60 F_{254} (Merck 5549, USA).

Chemicals. Phenols were purchased from SISCO Chem Industries, Bombay, India. Phosphorus oxychloride was purchased from Spectrochem private limited Bombay, India. Zinc chloride was made anhydrous by fusion and then used. 6-Methylcoumarin [13](Aldrich, M3,620-3); 7-methylcoumarin [14](Aldrich, 22,032-9); ethyl 3-coumarincarboxylate [15](Aldrich, 39,080-1) and rhodamine 123 hydrate (R123)(Aldrich, 28394-0) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). (±)-Verapamil (Sigma, V 4629) as calcium channel blocker (3,5) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

General method of preparation of 4-hydroxycoumarins [1-6]. 4-Hydroxycoumarins [1-6] were prepared according to the methods of Shah and Bose (6,7). Phenol or substituted phenol (0.1 M) and malonic acid (0.1 M) were added to a mixture of 30 mL phosphorus oxychloride and 36 g anhydrous zinc chloride, which was preheated to 60°C and the reaction mixture was heated on a water bath at 70°C for 16-36 hours. After the reaction had finished, it was cooled and decomposed with crushed ice,

the product was crystallized and was filtered, followed by washings with water. The product was dissolved in 10% sodium carbonate, warmed if neccessary and filtered. The filtrate was slowly acidified with 3 M HCl till complete precipitation, then filtered, washed with water, dried and recrystallized from EtOH or AcOH (Scheme 1).

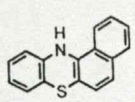
The following compounds were obtained:

a) *4-Hydroxycoumarin [1].* Yellow powders (EtOH). mp:209-210°C (Lit. 6: mp 201-203°C).
b) *6-Methyl-4-hydroxycoumarin [2].* Yellow powders (EtOH). mp:251-253°C (Lit. 8: mp 240°C).
c) *5,7-Dimethyl-4-hydroxycoumarin [3].* White powders (AcOH). mp:210-211°C (Lit. 7: mp 210-211°C).
d) *5,8-Dimethyl-4-hydroxycoumarin [4].* Yellow powders (EtOH). mp:261-262°C (Lit. 9. mp 261-262°C). ^{1}H-NMR (60 MHz, TFA) δ: 2.47 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 6.22 (s, 1H, H-3), 7.41 (br s, 1H, ArH), 7.60 (br s, 1H, ArH).
e) *7,8-Dimethyl-4-hydroxycoumarin [5].* Yellow powders (EtOH). mp:236-237°C (Lit. 7: mp 236-237°C).
f) *6-Chloro-7-methyl-4-hydroxycoumarin [6].* Yellow powders (EtOH). mp:229-230°C (Lit. 7: mp 229-230°C).

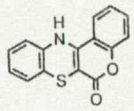
General method of preparation of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [7-12]. 6,12-Dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones **[7-12]** were prepared according to the method of K.Tabakovic *et al* (10). A mixture of appropriate 4-hydroxycoumarin **[1-6]** (0.01M) and 2-aminothiophenol (0.01M) in 25-30 mL DMSO was stirred and heated at 140-150°C for 10-13 hours. At the stage when the reaction mixture became dark, heating was stoped and the mixture slowly distilled in an excess of approximately 15-17 mL DMSO at atmospheric pressure. The reaction mass was then cooled to obtain dark colored crystallized product. This product was washed with MeOH and purified by three time washings and its purity was checked by TLC using CH_2Cl_2-MeOH (10:1) system (Scheme 1).

The following compounds were obtained:

a) *6,12-Dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [7].* Reddish brown crystals. mp:337-340°C (MeOH) (Lit. 10: mp 337-340°C).
b) *2-Methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [8].* Dark orange crystals. mp:340°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.40 (s, 3H, CH_3), 6.82-6.88 (m, 2H, ArH), 6.95-7.20 (m, 2H, ArH), 7.26 (d, 1H, *J*=8.2 Hz, ArH), 7.44 (d, 1H, *J*=8.5 Hz, ArH), 7.92 (s, 1H, ArH), 8.94 (s, 1H, NH). IR (Nujol) cm^{-1}: 3350 (NH), 1655 (C=O). MS m/e: 281 (M+, 100%). High-resolution MS: Calcd for $C_{16}H_{11}NO_2S$: 281.0511. Found: 281.0506.
c) *1,3-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [9].* Green crystals. mp:290°C (MeOH). yield: 52%. ^{1}H-NMR (DMSO-d6) δ: 2.33 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 6.68-6.73 (m, 1H, ArH), 6.79-6.85 (m, 1H, ArH), 6.87-6.98 (m, 3H, ArH), 6.98-7.40 (m, 1H, ArH), 10.10 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3300 (NH), 1630 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0653.
d) *1,4-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [10].* Light green crystals. mp: 170°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.26 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 6.35-6.45 (m, 1H, ArH), 6.65-6.76 (m, 1H, ArH), 6.80-7.10 (m, 3H, ArH), 7.28-7.37 (m, 1H, ArH), 7.96 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3300 (br, NH), 1670 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0654.
e) *3,4-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [11].* Orange crystals. mp:>300°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.24 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 6.82-6.87 (m, 2H, ArH), 6.96-7.02 (m, 2H, ArH), 7.24 (d, 1H, *J*=8.2 Hz, ArH), 7.86 (d, 1H, *J*=8.2 Hz, ArH), 8.94 (s, 1H, NH). IR (Nujol) cm^{-1}: 3340 (NH), 1655 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0667.
f) *2-Chloro-3-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4] benzothiazin-6-one [12].* Reddish brown crystals. mp:328°C (MeOH). yield: 45%. ^{1}H-NMR (DMSO-d_6) δ: 2.40 (s, 3H, CH_3), 6.83-6.87 (m, 2H, ArH), 6.94 (d, 1H, *J*=7.6 Hz, ArH), 6.97-7.01 (m, 1H, ArH), 7.42 (s, 1H, ArH), 8.25 (s, 1H, ArH), 8.98 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3350 (NH), 1660 (C=O). MS m/e: 315 + 317 (3:1) (M^+, 100%: 38.6%). High-resolution MS: Calcd for $C_{16}H_{10}{}^{37}ClNO_2S$: 315.0121. Found: 315.0129. Calcd for $C_{16}H_{10}{}^{37}ClNO_2S$: 317.0091. Found: 317.0085.



Benzo[*a*]phenothiazine



6,12-dihydro-1-benzopyra[3,4-*b*][1,

4]benzothiazin-6-ones[**7-12**]

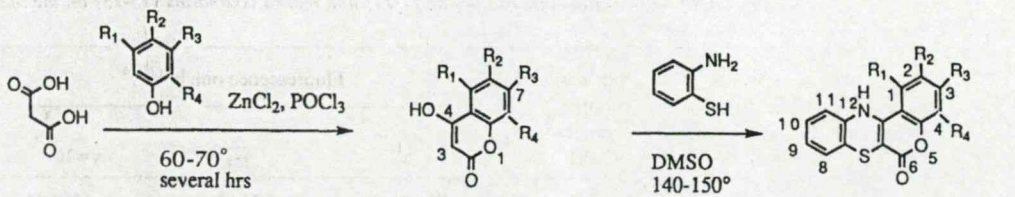
Figure 1. *Benzo[a]phenothiazine and 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [7-12].*

Biological evaluation

Measurement of antiplasmid activity. The F'lac plasmid of *E. coli* served as a convenient model in this study because the plasmid carrying colonies were easily differentiated from the plasmidless colonies on a simple eosinum methylene blue (EMB)-differential media. The antiplasmid activity of test compounds was measured on *E. coli* K12 LE140 F'lac strain in MTY broth which contained various concentrations of test compounds (11,12). After 24 hours of incubation at 37°C, various dilutions of samples were plated on EMB agar. The lactose-positive (plasmid carring) colonies and lactone-negative (plasmidless) colonies were counted (Table I).

Tissue cultures. L5178 mouse T cell lymphoma and its MDR1/A retrovirus transfected derivative were provided from Prof. Aszalos (Table II).

Cell and fluororescence uptake. MDR1/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the *mdr* phenotype. The L5178 MDR cell line, and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2 x 10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquots to Eppendorf centrifuge tubes. Then, the tested compounds were added in 2.0 μL of the 2.0 mg/mL stock solution and the samples were incubated for 10 minutes at room temperature. Then, 50 μL rhodamine 123 (R123) as indicator was added to the samples (5.2 μM final concentration) and the cells were incubated for a further 20 minutes at 37°C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument (cell sorter). (±)Verapamil was used as the positive control in the R123 accumulation experiments (13,14). The R123 accumulation was calculated from fluorescence of one height value using the 2nd equation $y=10^{\frac{x}{256}}$ (Table II). In the case of logarithmic transformation, the 1024 digital channels were switched to one decade at each 256 (=2^8) channels. Then, the percentage of control mean of the



1: R_1=H, R_2=H, R_3=H, R_4=H

2: R_1=H, R_2=CH_3, R_3=H, R_4=H

3: R_1=CH_3, R_2=H, R_3=CH_3, R_4=H

4: R_1=CH_3, R_2=H, R_3=H, R_4=CH_3

5: R_1=H, R_2=H, R_3=CH_3, R_4=CH_3

6: R_1=H, R_2=Cl, R_3=CH_3, R_3=H

7: R_1=H, R_2=H, R_3=H, R_4=H

8: R_1=H, R_2=CH_3, R_3=H, R_4=H

9: R_1=CH_3, R_2=H, R_3=CH_3, R_4=H

10: R_1=CH_3, R_2=H, R_3=H, R_4=CH_3

11: R_1=H, R_2=H, R_3=CH_3, R_4=CH_3

12: R_1=H, R_2=Cl, R_3=CH_3, R_3=H

Scheme 1. *Synthesis of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin -6-ones[7-12].*

fluorescence intensity was calculated for parental and *mdr* cell lines, compared to untreated cells. An activity ratio was calculated by the following equation (13,15)(Table II):

$$\text{Ratio} = \frac{(mdr \text{ treated}/mdr \text{ control})}{(\text{parental treated}/\text{parental control})}$$

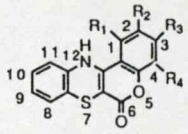
Results and Discussion

Antiplasmid activity by elimination of F'lac. Antiplasmid activity by elimination of F'lac was tested and only one compound the 7-methylcoumarin [**14**] had a remarkable effect, while three other derivatives [**8**], [**11**] and [**12**] had slight effect. The other coumarins [**7, 9, 10, 13, 15**] were ineffective (Table I).

The activity of 7-methylcoumarin [**14**] is probably related to the electrophilic-superdelocalization induced by methyl substitution at 7 position. If this correlation were true then we would expect similar effects in case of compounds [**9, 11, 12**]; however, these compounds were weak. The effect of [**9**] was reduced by methylation at position 1 due to a compensating electronic distribution.

mdr Reversal on tumor cells. The effect of 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones [**7-12**] and coumarins [**13-15**] was tested on the *mdr* reversal on tumor cells. The coumarins used in this study were slightly effective (Table II). Three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones of [**8**] (Fluorescence activity ratio, 1.56), [**9**] (Fluorescence activity ratio, 1.43) and [**10**] (Fluorescence activity ratio, 1.11) at 20 μg concentration had a moderate activity on *mdr* reversal (Fluorescence activity ratio>1). However, three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones [**7,11,12**] (Fluorescence activity ratios, 0.57,

Table I. *Antiplasmid activity of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [7-12] and related coumarins [13-15].*



Compd's No.	R_1	R_2	R_3	R_4	Elimination of F'lac (μg/mL)	Antiplasmid effect (%)
7	H	H	H	H	200	0
8	H	CH_3	H	H	>200	0.05/at 160 μg
9	CH_3	H	CH_3	H	200	0
10	CH_3	H	H	CH_3	200	0
11	H	H	CH_3	CH_3	200	0.1/at 160 μg
12	H	Cl	CH_3	H	>200	0.9/at 180 μg
13 (6-methylcoumarin)					200	0
14 (7-methylcoumarin)					95	32.0/at 70 μg
15 (ethyl 3-coumarin-hyphen carboxylate)					200	0

Table II. *The effect of 6,12-dihydro-1-benzopyrano[3,4-b][1-4]benzothiazin-6-ones [7-12] and related coumarins [13-15] on the mdr reversal on L-5178 tumor cells with multidrug resistance.*

Compound's number	Concentration (μg)	Forward scatter count (cell size)	Side scatter count (granulation of cell)	Fluorescence one height[a]			Fluorescence activity ratio[c]
				x[b]	$\frac{x}{256}$	$y=10^{\frac{x}{256}}$	
par[d]	control	501.31	335.65	781.9520	3.0545	1133.80	-
mdr + R123[e]	control	552.47	385.44	326.0672	1.2737	18.78	1.00
(±)-veraparnil[f]	8 μg	540.50	381.20	563.8161	2.2024	159.37	8.49
7	2 μg	551.31	375.07	246.9632	0.9647	9.22	0.49
	20 μg	533.43	400.03	263.5264	1.0294	10.70	0.57
8	2 μg	557.39	375.09	263.6261	1.0298	10.71	0.57
	20 μg	541.54	388.52	375.3984	1.4664	29.27	1.56
9	2 μg	553.48	379.05	298.3680	1.1655	14.64	0.78
	20 μg	544.72	375.61	366.0288	1.4298	26.90	1.43
10	2 μg	551.91	385.77	291.9680	1.1405	13.82	0.74
	20 μg	543.69	380.65	337.7408	1.3193	20.86	1.11
11	2 μg	540.58	368.60	235.6851	0.9206	8.33	0-44
	20 μg	553 07	374.34	269.1072	1.0512	11.25	0.60
12	2 μg	550.50	386.64	251.5712	0.9827	9.61	0.51
	20 μg	544.76	382.16	275.4304	1.0759	11.91	0.63
13	2 μg	558.61	396.64	254.8736	0.9956	9.90	0.53
	20 μg	559.92	389.56	252.8512	0.9877	9.72	0.52
14	2 μg	559.13	393.86	265.9840	1.0390	10.94	0.58
15	2 μg	522.56	397.48	264.1408	1.0318	10.76	0.57
	20 μg	513.96	371.64	275.8144	1.0774	11.95	0.64

[a]Ref.15. [b]x: Measured fluorescence value at linear scale [μg, uptake of R123].
[c]The R-123 accumulation was calculated from fluorescence of one height value using 1st equation $=10^{\frac{x}{256}}$; the fluorescence activity ratios were calculated according to the formula given below;

$$\text{Ratio} = \frac{(\textit{mdr}\text{ treated}/\textit{mdr}\text{ control})}{(\text{parental treated/parental control})}$$

[d]par: parental without multidrug resistant gene; [e]*mdr*: parental with multidrug resistant gene.
[f](±)-verapamil: a control for *mdr* reversal.

0.60 and 0.63, respectively) without methyl or benzo group at positions 1 or 2 together reduced the rhodamine accumulation in tumor cells, probably by inducing the efflux pump mechanism or by causing a direct membrane injury (Table II). Position 4 must be free or low in electron density for the *mdr* reversal effect. The one exception is [**10**], in which the substitutions at positions 1 and 4 neutralized each other.

The ineffectivity of the compounds was probably correlated with the lower or reduced cell size, however, granulation did not change remarkably in the cells. There was no toxic effect in the applied concentration, and the cell size did not change in flow cytometry.

References

1 Motohashi N: Test for antitumor activities of phenothiazines and phenoxazines. (in Japanese) Yakugaku Zasshi *103:* 364-371, 1983; Chem Abst *99:* 231v, 1983.

2 Motohashi N, Sakagami H, Kamata K and Yamamoto Y: Cytotoxicity and differentiation-inducing activity of phenothiazine and benzo[*a*]phenothiazine derivatives. Anticancer Res *11:* 1933-1938, 1991.

3 Szabó M, Csiszár J, Rausch H, Molnár J and Motohashi N: Influence of benzo[*a*]phenothiazines on the element content of two tobacco tissue cultures differing in hormone requirement. Anticancer Res *17:* 2049-2056, 1997.

4 Shah A, Naliapara Y, Surcja D, Motohashi N, Kurihara T, Kawase M, Satoh K, Sakagami H and Molnár J: Biological activity of 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones. Anticancer Res *18:* in press, 1998.

5 Zhao H, Neamati N, Hong H, Mazumder A, Wang S, Sunder S, Milne GWA, Pommier Y and Burke TR: Coumarin-based inhibitors of HIV integrase. J Med Chem *40:* 242-249, 1997.

6 Shah VR, Bose JL and Shah RC: New synthesis of 4-hydroxycoumarins. J Org Chem *25:* 677-678, 1960.

7 Shah AK, Bhatt NS, Raval RV and Thakor VM: Synthesis of 4-hydroxycoumarins. Curr Sci *53:* 1241-1242, 1984.

8 Ziegler E and Junek H: A new synthesis of 4-hydroxycoumarin and its derivatives. Monatsch Chem *86:* 29-38, 1955.

9 Shah A: Ph.D. Thesis, Saurashta University, Rajkot, 1982.

10 Tabakovic K, Tabakovic I, Trkovnik M, Juric A and Trinajstic N: Studies on novel heterocyclic ring systems. Reaction of 4-hydroxycoumarin with *o*-aminobenzaldehyde and 2-mercaptoaniline. J Heterocyclic Chem *17:* 801-803, 1980.

11 Motohashi N, Sakagami H, Kurihara T, Csuri K and Molnár J: Antiplasmid activity of phenothiazines, benzo[*a*]phenothiazines and benz[*c*]acridines. Anticancer Res *12:* 135-140, 1992.

12 Molnár J, Kiraly J and Mandi Y: The antibacterial action and R-factor inhibiting activity by chlorpromazine. Experimentia *31:* 444-445, 1975.

13 Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S and Aszalos A: The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int J Cancer *54:* 456-461, 1993.

14 Kessel D: Exploring multidrug resistance using rhodamine 123. Cancer Commun *1:* 145-149, 1989.

15 Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J and Rosenberg SA: Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res *54:* 1766-1771, 1994.

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V.

Nitric Oxide Production and MDR Expression by Human Brain Endothelial Cells

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Abstract. *The endothelium both initiates and responds to a cascade of events triggered by cytokines. Enhanced formation of NO, especially by inducible nitric oxide- synthase (i NOS), is largely stimulated by tumor necrosis factor (TNF). Nitrogen oxides are reactive intermediate molecules functioning in neural transmission, and vasodilatation. The aim of our study was to investigate the effect of TNF and Staphylococcus aureus, a TNF inducing agent on the NO production of brain endothelial cells in vitro. The effect of the same agent was investigated on the MDR expression of endothelial cells. Both TNF and Staphylococcus aureus resulted in enhanced NO production. Western blot analysis showed enhanced expression of iNOS, which could be inhibited by pentoxifylline, an inhibitor of TNF synthesis. Flow cytometric analysis revealed that the brain capillary endothelial cells exerted P-glycoprotein expression, which was not influenced by TNF. However, the mdr function itself in these cells was decreased by TNF. Cultured endothelial cells are excellent tools for the investigation of the possible connection between the NO production and MDR function, and for the estimation the effect of different agents influencing these activities, which might be important in blood-brain barrier function.*

Brain endothelial cells possess a unique structure which is responsible for their anatomical barrier properties. Substances entering or leaving the brain must pass through the endothelial cells. The multidrug resistance protein in brain capillary endothelium may be one of the functional components of the blood brain barrier, functioning as an active efflux pump (1).

On the other hand, cytokines can penetrate the blood brain barrier at specific sites. One of the most important actions of the tumor necrosis factor (TNF), a pluripotent, proinflammatory cytokine, is that it can increase the permissivity of the barrier (2). It is interesting that endothelial cells (EC) both initiate and respond to a cascade of events triggered by cytokines. Enhanced formation of niric oxide (NO), especially by the inducible nitric oxid synthase (iNOS), is largely stimulated by TNF (3). Nitric oxides are reactive intermediate molecules functioning in vasodilatation, or even in neural transmission (3).

The aim of our studies was to investigate the TNF and *Staphyloccus aureus*, as a TNF inducing agent on the NO production of brain endothel cells (EC) *in vitro*. The effect of the same agents were studied on the MDR expression and MDR function in cultured endothelial cells.

Materials and Methods

Cells. Brain capillary endothelial cells of a BB19 cell line were cultured in monolayers, in RPMI medium supplemented with 10% FCS and 10% human AB serum, and 50 μg/ ml endothelial growth factor (SIGMA)

Measurement of nitrite/nitrate concentrations. For the determiation of total nitrite/ nitrate concentrations in cell supernatants, nitrate was first converted to nitrite, by incubation with nitrate reductase and NADPH (Total NO kit, Caymann Chemicals, U.S.A), and then nitrite was measured by the Griess reaction (4). Adsorbance at 540 nm was measured by use of a StaFax ELISA plate reader. The nitrite present in each well was quantified by a comparison with a standard curve of serial dilution of a 10 mM solution of sodium nitrite.

Western blot analysis. For analysis of iNOS content of EC, 3×10^6 EC were cultured for 18 h in the presence or abscence of 1000 IU/ ml of hrTNF (Genzyme) or with 10^8 heat killed *S. aureus*. Cell lysates in 2X Laemmli reducing sample buffer were boiled for 5 min at 100°C. Samples were loaded onto SDS polyacrylamide gel electrophoresis consisting of 10 % running gel. After electrophoresis, the proteins were transferred to nitrocellulose and probed with a 1:1000 dilution of rabbit anti-iNOS polyclonal antibody (Affinity, U.K.) followed by a 1:1000 diluton of goat anti- rabbit IgG conjugated to HRP (Bio Rad). The color was developed with diaminobenzidine (DAB, SIGMA) and hydrogen peroxide.

Indirect immunofluorescence analysis of P glycoprotein. Endothelial cells (2×10^6 cells in 50 μl) were incubated for 45 min on ice with 50 μl of a 1:50 dilution of Monoclonal antibody C219, specific for P-glycoprotein

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Key Words: Multidrug resistance, endothelial cells, TNF, nitric oxide.

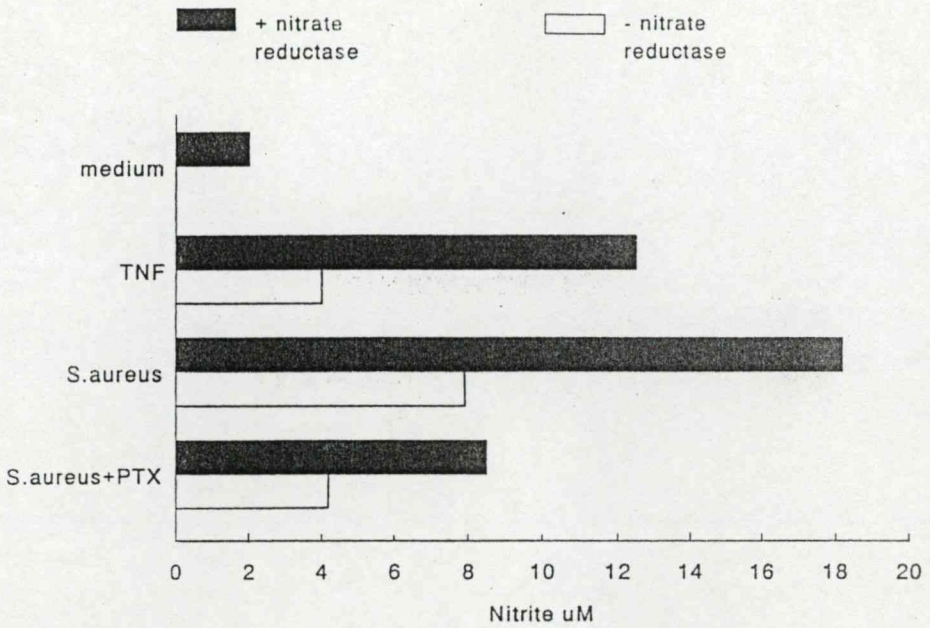

Figure 1. *NO production by human brain endothelial cells. Endothelial cells grown in monolayers were incubated in the presence of 1000 1U/ml rhTNF, or 10^8/ml heat killed S. aureus respectively, and in the presence of the S. aureus with 100 µg/ml pentoxiphylline (PTX), for 18 hours. Nitrite, (NO2) was measured in culture supernatants by the Griess reaction, with the Total NO kit (Caymann Chemicals), where nitrate was first converted to nitrite by incubation with nitrate reductase. Thereafter, nitrite was measured by the Griess reaction, and determined by measuring absorbance at 550 nm in a STATFAX ELISA reader.*

(ID Labs). Cells were washed three times with ice-cold PBS containing 1 % FCS and stained for 45 min on ice with a 1:200 dilution of fluoresceinated $F(ab')_2$ fragments of goat anti-mouse Ig (DAKO). Cells were washed three times with ice-cold PBS- 1% FCS and fluorescence analysis was performed with FACStar plus (Becton- Dickinson) at 488 nm excitation. Background fluorescence was measured by incubatlon of the cells with buffer only, instead of with C219 mAb.

Fluorescence uptake assay. Brain endothelial cells grown in monolayer were adjusted to a concentration of 2×10^6/ ml after trypsinisation, and resuspended in serum free medium. Test compounds were added to the cell suspensions and the samples were incubated for 10 minutes at room temperature. then 50 µM of the indicator Rhodamine 123 was added to the samples and the cells were incubated for a further 20 minutes at 37°C, washed twice and resuspended in 0.5 ml PBS for analysis. The fluorescence of cell population was measured by flow cytometry using a Beckton Dickinson FACScan instrument.

Results

Effect of TNF on NO production by endothelial cells. Endothelial cells were incubated with 1000 U/ml TNF or in the presence of heat killed *St. aureus* for 18 hours thereafter, the NO production was estimated. Nitrite, (NO2), a rapidly formed stable metabolite of NO, was measured in culture supernatants by the Griess reaction, with the Total NO kit (Caymann Chemicals), Nitrate and nitrite are the primary oxidation products of NO, reacting with water. Therefore,the total nitrite concentration in the supernatants was used as an indicator of changes in NO production. Nitrate was first converted to nitrite, by incubation with nitrate reductase. Thereafter, nitrite was measured by the Griess reaction, and determined by measuring absorbance at 540 nm in a STATFAX ELISA reader.

The detection of nitrite content of the supernatant was much more sucsessful if the nitrite reductase was applied (Figure 1). TNF induced nitrite production, which was more pronounced if the inducing agent was the heat killed *S. aureus*. This induction could be inhibited by the addition of pentoxifylline (PTX, 100 µg/ml) which is able to inhibit the TNF production (5). So we suppose that the NO induction by *Staphylococcus* is due to the TNF inducing abilty of the bacteria.

Detection of iNOS by Western blot analysis. Not only the the NO production also but the expression of the protein of the inducible NO synthase (iNOS) itself could be detected in endothlial cells, by Western blot technique (Figure 2).

Using a polyclonal antibody which specifically detect the protein of iNOS of a molecular weight of 130 kD, a positive band was appeared with the positive controls provided by the manufacturer (Figure 2. upper compartment). The results shown in the lower compartment of Figure 2. demonstrate the same band at the same Mw position when applying endothelial cells. After incubation of EC with TNF, the inducible enzyme protein could be detected, and also after the incubation of cells with *S. aureus.* However, the effect of pentoxifylline proved to be inhibitory even on the expression of the enzyme. In addition, incubation of endothelial cells with granulocytes also led to the expression of iNOS. Note

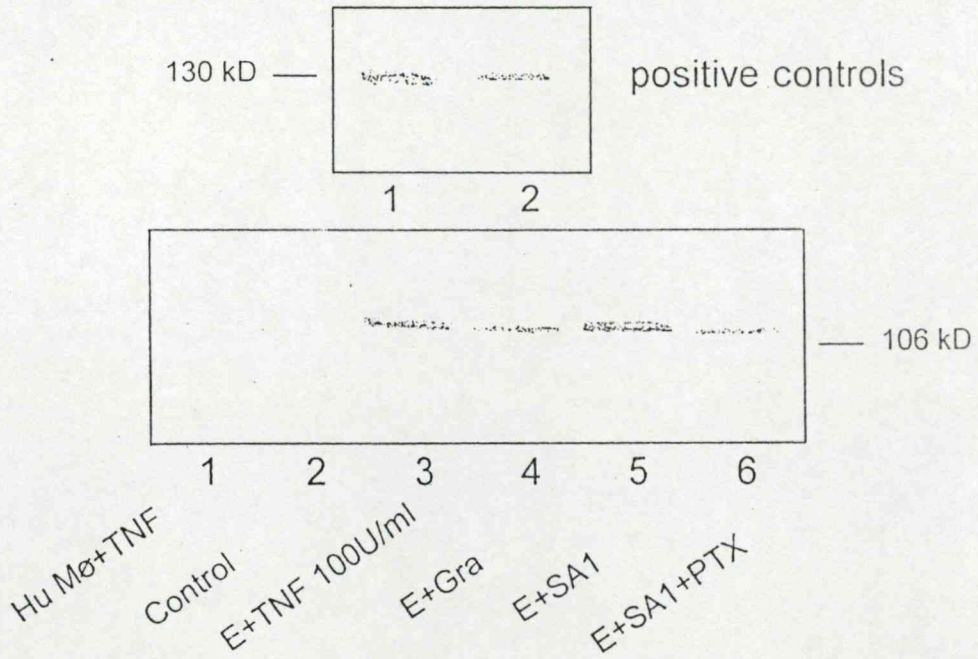

Figure 2. *iNOS expression by brain endothelial cells (EC). EC lysate protein separated by 10 % SDS PAGE. EC were cultured for 18 hours in the presence (lane 3) or absence (lane 2) of 1000IU/ml of hrTNF (Genzyme) or with 10^8 heat killed S. aureus (lane 5), or with 10^6/ml human granulocytes (lane 4) respectively. Lane 6: 100 µg/ml pentoxifylline(PTX) were added together with S. aureus. Western blot was incubated with antibody to iNOS. Bound antibody was visualized by horse radish peroxidase mediated color development of diaminobenzidine.*

that we could not detect this enzyme in human macrophages (lane 2).

Next we investigated the effect of these nitric oxide inducing agents on the expression and function of MDR in endothelial cells.

The expression of the P-glycoprotein. This was evaluated with monoclonal antibodies directed to the intracellular epitope of the protein. Endothelial cells were investigated by means of indirect immunoflourescency and flow cytometry analysis, (Figure 3). Flow cytometry analysis revealed the presence of the P-glycoprotein on the cells after staining with the monoclonal antibody C 219. TNF and *Staphylococcus aureus* did not significantly influence the mdr expression, only a slight increase can be seen on the histogram.

Effect of TNF on mdr function. Modulation of the mdr function itself was investigated by the rhodamine exclusion test. The fluorescence intensity depending on the rhodamine intake was measured by flow cytometry (Table I). There was a moderate increase in fluorescence intensity when cells were incubated in the presence of TNF, which might be due to the decrease of the mdr function. The most pronounced effect was observed in the case of treatment with 7,8 dioxo

Table I. *Rhodamine 123 accumulation by brain endothelial cells. Flow cytometric analysis was performed as described in Materials and methods.*

Treatment	F1-1
control	1269.29
TNF 10^3U/ml	1477.63
7,8 O_2 CPZ 1ug/ml	1414.06

Chlorpromazine which proved to be an effective inhibitor of mdr in other cell systems (6).

Discussion

The gene responsible for multidrug resistance, termed mdr1, encodes a membrane glycoprotein (P-glycoprotein) that acts as a pump which transports various drugs out of the cell (7). The amount of P- glycoprotein expression was found to be elevated in drug resistant cancers (8). In addition to tumor cells, the P-glycoprotein is also expressed in various normal tissues such as capillary endothelium in brain (9). P-

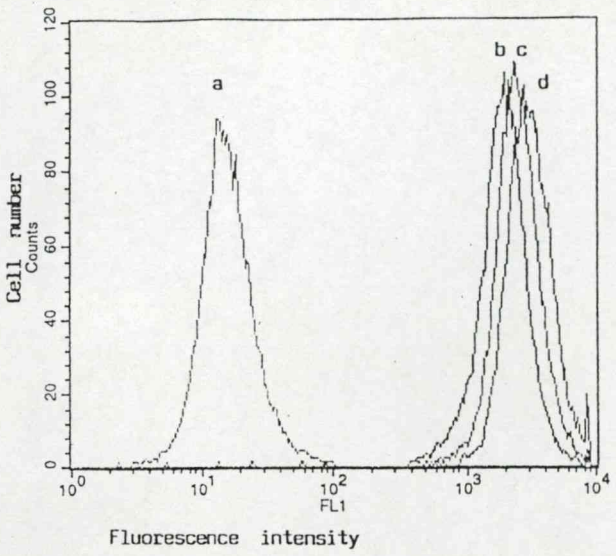

Figure 3. *Effect of TNF and S. aureus on P-glycoprotein expression of human brain endothelial cells. EC were incubated for 18 hours in the presence of (b) no stimulant, (c) 1000 IU/ml rhTNF, or (d) S. aureus. Cells were incubated with C219 mAb then with F(ab')2 anti-mouse FITC-conjugated antibody. The background fluorescence of cells incubated with medium only is shown in graph (a). The result is a representative of three independent experiments with similar results.*

glycoprotein in brain capillary endothelium may be one of the functionar components of the blood brain barrier.

The mAb C219 used in this study is able to detect P-glycoprotein on cultured human brain endothelial cell line.

Brain endothelial cells are also one of the sources of iNOS, which can be expressed following the exposure to various inflammatory mediators such as cytokines (3). In addition, TNF is able to increase the blood brain barrier permeability (2), which in turn might be mediated through nitric oxide and cyclic nucleotide formation (10). There is no data as yet about the connection of mdr function and the roduction of nitric oxide by brain endothelial cells in the presence of different drugs. We suppose therefore that human endothelial cell cultures might be a good tool for the *in vitro* investigation the effect of different drugs influencing these functions which are essential in the blood brain barrier, or even in cancer therapy of tumors originating from the central nervous system.

Acknowledgements

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References

1 Jette L, Pouliot JF, Murphy GF, Beliveau R: Isoform I (mdr3) is the major form of P-glycoproteinexpressed in mouse brain capillaries. Biochem J *305:* 761- 766, 1995.

2 Abraham Cs, Deli M, Joo F, Megyeri P, Torpier G: Intracarotid tumor necrosis factor administration increases the blood brain barrier permeability in cerebral cortex. of the newbom pig. Neurosci Lett *208:* 85-88, 1996.

3 Nathan C: Nitric oxide as a secretory product of mammalian cells. FASEB J *6:* 3051-3064, 1992.

4 Tracey WR, The J, Carter G: Lipopolysacharide induced changes in plasma nitrite and nitrateconcentrations in rats and mice, pharmacological evaluation of nitric oxide synthase inhibitors. J Pharm Exp Ther *272:* 1011-1015, 1995.

5 Doherty GM, Jensen JC, Alexander HR, Buersh, CM, Norton JA: Pentoxifylline suppression of tumor necrosis factor gene transcription. Surgery *110:* 193-198, 1991.

6 Molnar J, Hever A, Fakla I, Fischer J, Ocsovszki I, Aszalós A: Inhibition of the transport function of membrane proteins by some substituted phenothiazines in E.coli and multidrug resistant tumor cells. Anticancer Res *17:* 481-486, 1997.

7 Roninson IB: The role of the mdrl (P-glycoprotein) gene in multidrug resistance in vivo and in vitro Biochem. Pharmacol *43:* 95- 102, 1992.

8 Nooter K, Stoter G: Molecular mechanisms of multidrug resistance in cancer chemotherapy. Path Res Pract *192:* 768-780, 1996.

9 Tsuruo T: Biochemical function of P- glycoprotein and implication for therapy. *In:* The mechanism and New Approach of Drug Resistance in Cancer Cells. Eds Miyazaki T, Takau F. Sakurada. Elsevier, p 81- 91. 1993.

10 Murata J, Betz Corradin S, Janzer RC, Jeanneret L: Tumor cell suppresscytokine induced nitric oxide (NO) production in cerebral endothelial cells. Int J Cancer *59:* 699-705, 1994.

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VI.

The Inhibition of SOS-Responses and MDR by Phenothiazine-Metal Complexes

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Abstract. *The gene of multidrug resistance (mdr) is inducible by different environmental stresses (SOS gene). We tested the inhibitory action of some new metal complexes of phenothiazines on megacin encoding bacterial gene induced by mitomycin-C as an example of "SOS induction" and on efflux pump of mouse lymphoma cells. The interaction of compounds to DNA was measured by thermal stability of DNA. It was found that metal co-ordination complexes of trifluoperazine (TFP) and chlorpromazine (CPZ) added before mitomycin administration have an inhibitory action on megacine induction. The TFP-V(IV) complex was effective at a lower concentration than TFP alone. The inhibitory effect of some metal co-ordinating complexes (TFP-Cu(II) and TFP- V(IV)) exceeded the action of TFP alone on efflux pumps. We propose that these compounds can form a complex with the regulatory protein or DNA resulting in the inhibition of SOS response and inhibit the mdr function by inactivating the P-glycoprotein as well.*

The multidrug resistance gene can be considered as a member of the family of stress-induced genes. Due to some similarities, the mdr gene promoter can also be activated by various environmental stresses, carcinogens and anticancer drugs. Therefore, it was suggested that the mdr gene promoter can be a target for stress-induced gene regulation (**1, 2, 3, 4,**). Bearing this in mind, it is not unlikely that phenothiazines (known as mdr efflux pump reversal compounds) can stabilise the drug sensitivity in bacteria and cancer cells. It is possible that phenothiazines reduce the cancerous transformation (and mutation rate of bacteria) *via* the inhibition of SOS functions. It is known that mitomycin-C induces SOS response in bacteria either by causing DNA damage due to intercalation or by inhibition of DNA replication. The stabilisation of repressor protein (lex-A)-DNA complex blocks the SOS response. Some tricyclic compounds (*e.g.* TFP and CPZ) are able to inhibit both the inducing effect of mitomycin-C and the efflux pump of mdr (**5, 6**). It was proposed, that metal ions, coordinating to the hetero atoms of the middle ring, induce some changes in the structure of the molecule and in the binding features to DNA or the repressor protein of the SOS gene promoter and the P-glycoprotein (by the efflux pump mechanism).To improve the effect of TFP and CPZ metal co-ordination complexes, - namely CPZ-Pt(II), CPZ-Sn(IV), TFP-Sn(IV), TFP-Cu(II), TFP-Ni(II), TFP-Pd(II) and TFP-V(IV) - were prepared and studied.

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Key Words: mdr, SOS, stress respons gene, thermal stability of DNA, phenothiazine, trifluoperazine, chlorpromazine, metal complex, mitomycin.

Materials and Methods

The phenothiazines (promethazine (Pz), CPZ, TFP) and the dimethyltin(IV)-dichloride were purchased from Aldrich, the di-n-butyltin(IV) oxide and the metal salts used were purchased from Fluka. The CPZ complexes with di-n-butyltin(IV) oxide and platinum(II) and the TFP complexes with di-n-butyltin(IV) oxide, copper(II), nickel(II) and vanadium(IV) in 1:1 ratio of the components were prepared by Nagy *et al* (**7**) .The complex of palladium(II) and TFP was prepared also in 1:1 ratio, by dissolving the metal chloride in concentrated HCl and adding the aqueous solution of the ligand. The complex precipitated immediately was filtered off and recrystallised from DMF solvent. The analytical data showed that complexes with 1:1 metal to ligand ratio have been formed.

Detection of SOS signal by megacin induction. The plasmid in *B. megaterium 216* encodes a bactericidal substance called megacine, but only when the plasmid DNA and the repressor protein complex are

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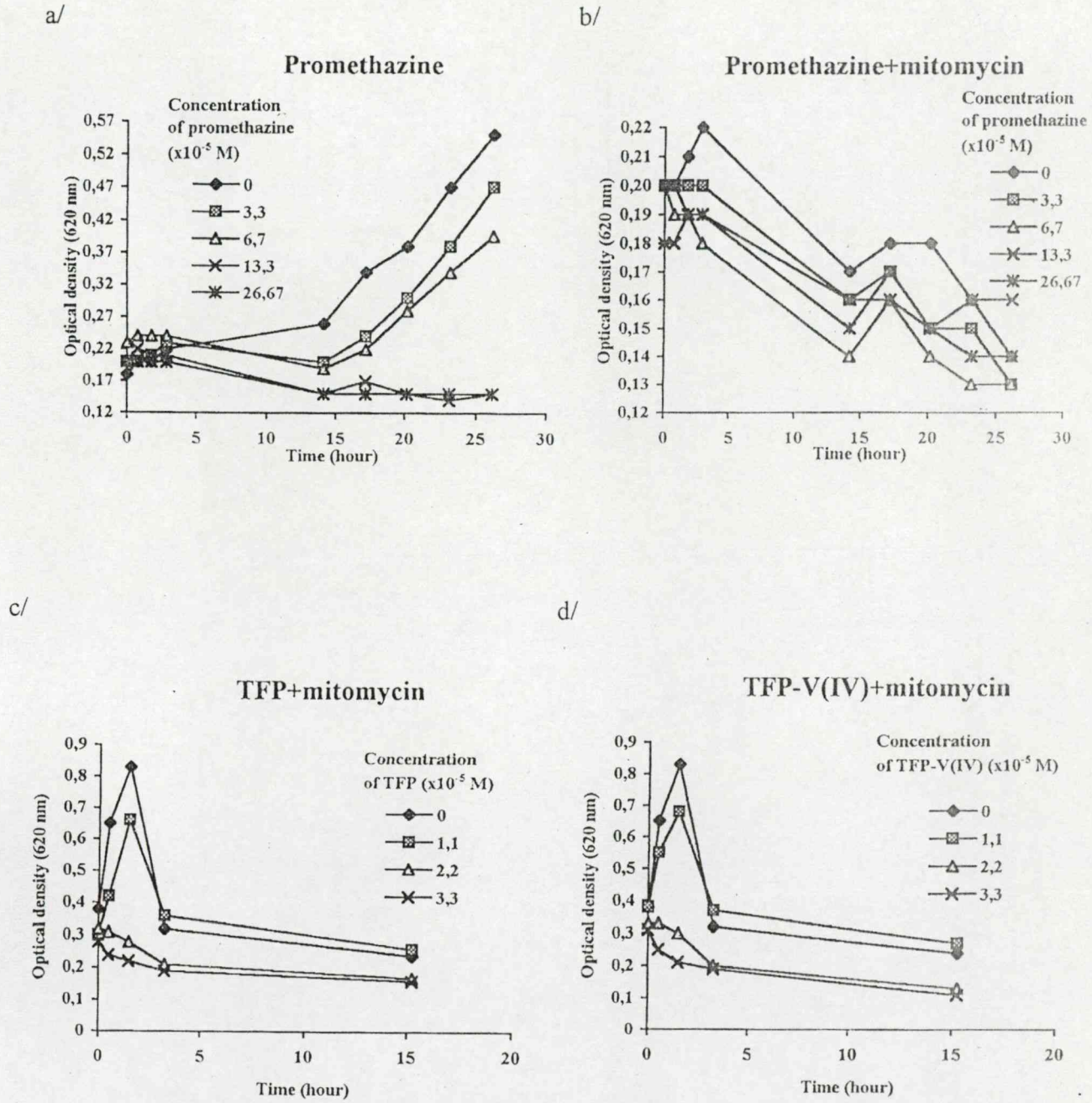

Figure 1. *Effect of pre-treatment of phenothiazines and their metal complexes on growth rate and induction of megacin by mitomycin-C in B. megaterium 216.*

separated by mitomycin-C. YT (yeast extract tryptone) medium: tryptone (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; K_2HPO_4, 1 g, NaCl, 5 g; distilled water, 1000 ml; pH 7.5. YP (yeast extract peptone) medium was prepared as described previously [12]. From a standing culture in YT medium incubated overnight 0.2 ml was transferred to 10 ml YT medium pipetted into a 100 ml Erlenmeyer flask. The flask was shaken in water bath at 37 °C. When an OD value of 0.25 had been reached (number of colony formers, $5x10^6$/ml average chain length, 4-5), the cultures were supplemented with phenothiazines and after 10 minutes with mitomycin (0.5 μg/ml) and the change in OD on further incubation was recorded. YP broth culture of B. megaterium KM was diluted 1 : 4 with YP medium and 1 ml was mixed with 1 ml molten (47 °C) nutrient agar and poured on a basic layer of the same medium. The basic layer was prepared with 1.5 % agar. The upper soft layer contained half of this concentration. Serial dilutions of the megacine containing lysates were prepared with saline containing 20 % YP broth. By means of a loop equal amounts of the serial dilutions were placed onto the surface of the indicator plates (highest titre was 128x). The reciprocal of the highest

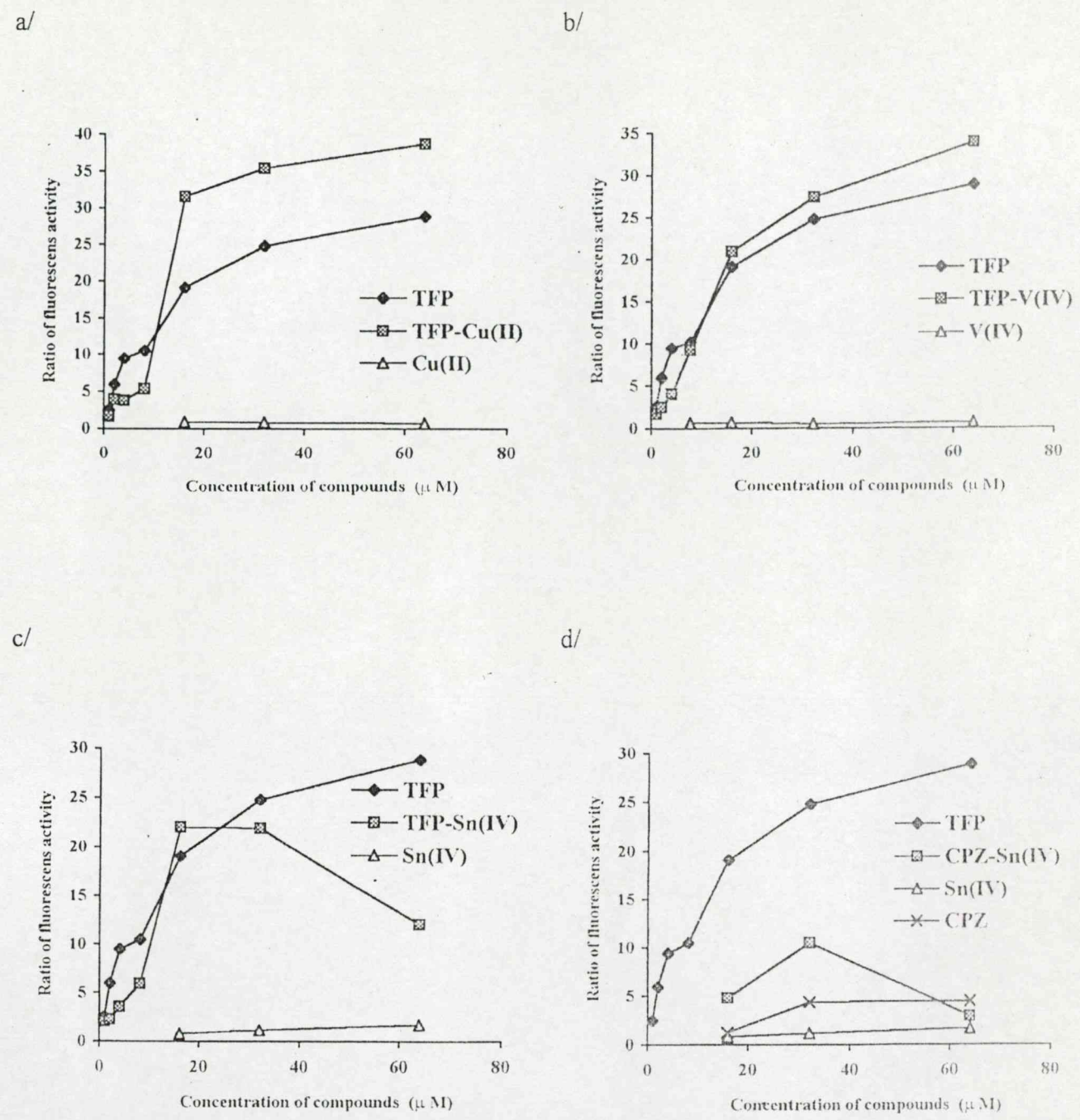

Figure 2. *Mdr reversal effect of phenothiazines and their metal complexes.*

dilution causing clear zones or lysing at least half of the bacteria at the corresponding site was regarded as one unit of megacin/ml **(8, 9).**

Reversal of efflux pump indicated by fluorescence uptake assay. The cells from L5178Y mouse T-cell lymphoma parent cell line and from its multidrug resistant subline L5178YvMDR (kindly provided by Prof Aszalos, FDA, Washington DC) were adjusted to a concentration of $2x10^6$/ml in McCoys 5A medium. This medium with 10% heat-inactivated horse serum, L-glutamine, penicillin/streptomycin was used for the L5178Y cell line. The medium for the L5 1 78vMDR cell line was supplemented with 60 ng/ml colchicine to maintain expression of the MDR phenotype. In 0.5 ml aliquots of cells were distributed into Eppendorf centrifuge tubes. To assay the test compounds they were added to cells and incubated for 10 minutes at room temperature, and then with indicator Rhodamine 123 (5.2 μM final concentration) for another 20 minutes at 37°C. After washing twice with PBS, the

Table I. *Effect of pre-treatment of metal complexes of phenothiazines on growth rate and induction of megacin in B. megaterium 216.*

		Concentration of compounds (10-5 M)			
		0	1,1	2.2	3.3
Compounds	Time of incubation (hour)	Optical density of culture (620 nm)			
TFP-Cu(II)					
	0	0.38	0.38	0,33	0,31
	0.5	0.65	0.55	0.36	0.24
	1	0.83	0,7	0.36	0.25
	3.25	0.32	0.25	0,2	0.25
	15.2	0.24	0.17	0.11	0.19
TFP-Pd(II)					
	0	0.38	0.36	0.35	0.25
	0.5	0.65	0.51	0.4	0.24
	1	0.83	0.64	0.36	0.23
	3.25	0.32	0.24	0.23	0.2
	15.2	0.24	0.17	0.17	0.17
TFP-Ni(II)					
	0	0.38	0.4	0,35	0.3
	0.5	0.65	0.58	0,48	0,3
	1.5	0.83	0.74	0.58	0.26
	3.25	0,32	0.32	0.24	0,22
	15.2	0.24	0.3	0,17	0,18
CPZ-Pt(II)					
	0	0,38	0,35	0,32	0,23
	0.5	0,65	0,41	0,3	0,18
	1.5	0,83	0,5	0,37	0,16
	3,25	0,32	0,26	0,27	0,14
	15.2	0,24	0,2	0,2	0,12

fluorescence of $1x10^4$ cells was measured by the flow cytometer (Beckton Dickinson. FACScan Instrument). The percentage of control mean fluorescence was calculated for untreated parental and multidrug resistant cells. Verapamil was used as a positive control. An activity ratio (R) was calculated by the following equation: R=(Mdr treated / Mdr untreated control).

DNA thermal stability studies. To study the interaction of compounds with DNA; one of the most convenient methods is to measure the thermal stability of the DNA (10). Melting can be monitored by an increase in absorbance (hyperchromic effect) that results from the disruption of base stacking. The mid-point of the thermal denaturation curve is referred to as the "melting temperature, (Tm)" of the DNA.

Table II. *Titre of megacin after the inhibition of mitomycin induction by phenothiazine and metal complexes.*

	Concentration of compounds (x10-5M)			
	0	1,1	2.2	3.3
Compounds	Titre of megacine			
TFP	128x	128x	8x	0x
TFP-V(IV)	128x	64x	4x	0x
TFP-Cu(II)	128x	128x	128x	1x
TFP-Ni(II)	128x	128x	128x	4x
TFP-Pd(II)	128x	128x	64x	0x
CPZ-Pt(II)	128x	128x	8x	8x

Highly polymerised type-I calfthymus DNA sodium salt was purchased from Sigma. Stock solutions of DNA and the phenothiazines were prepared as follows: DNA (10 mg) was suspended in 10 ml 0.03 M TRIS buffer containing 0.018 M NaCl adjusted to pH 7.0 and the solution was kept at 4°C for at least three days. The stock solution was diluted to the working conc immediately before use. The concentrations the DNA solutions were determined spectrophotometrically in terms of nucleotide phosphate and calculated from an extinction coefficient at 260 nm of 6600/M/cm for calf thymus DNA (11, 12). The phenothiazines and their metal complexes were dissolved in DMSO at a concentration of 10^{-3} M immediately before use.

Measurement was recorded on a Cary Varian Model 1E spectrophotometer using a Cary temperature controller connected to a Cary 1/3 multicell block. The solutions were allowed to equilibrate for 20 minutes before increasing the temperature and the temperature was then increased at a rate of 0.5 °C/minute. A cell containing DNA solution alone was always measured along side cells containing Drug/DNA mixtures to act as an internal standard. The blank cells in all measurements contained TRIS solution and all cells were stoppered with teflon caps. The mid point of the thermal denaturation profile of the solutions (Tm) was determined by calculating the average absorbance using the instruments thermal application software (13, 14).

Results

On adding promethazine alone to the culture of *B. megaterium 216* no megacine production was observed (Figure 1a). Promethazine pre-treatment was able to inhibit megacine production by inhibition mitomycin induction (Figure 2b). The Pz had concentration dependent antibacterial effect from 6.7 to $26.67x10^{-5}$ M (Figure 1a). The compounds had no effect on the production of megacine if the culture was treated by promethazine 10 minutes after mitomycin-C induction. The quantity of megacine was followed by the growth-lysis curve (depending on the time of incubation). As a result of the bactericidal effect of megacine in the culture the cells lysed. Similar effect was found in the case of CPZ. Since the membrane effects of Pz and CPZ cannot be excluded on bacteria, some newly prepared metal complexes were tested to avoid the direct membrane injury

caused by the phenothiazines. TFP one of the most potent phenothiazines had a concentration dependent inhibitory action on the megacine production (Figure 1c). The most effective complex of TFP was the vanadium complex (Figure 2d), while other metal-coordination complexes *e.g.* copper. nickel, palladium were less effective (Table I, II) showing that these three metals do not modify the biological action of TFP. The platinum complex of CPZ had similar effect to TFP (Table I, II). It was noted that the compounds do not neutralise the antibacterial action of preformed megacine (results not shown).

Induction of the mdr efflux pump can be considered as a unique type of SOS function; therefore, the phenothiazines and their metal co-ordination complexes were also tested on the activity of mdr of mouse lymphoma cells. None of the metal ions had a reversal effect on mdr at all! The TFP, TFP-Cu(ll), TFP-V(IV) and the TFP-Sn(IV) complex were the most effective on reversal of mdr of mouse lymphoma cells (Figure 2a, b, c,). The Ni(II) and Pd(II) complexes of TFP had activity, but less than the TFP itself had (data not shown). The less effective compounds were the Sn(lV) and Pt(ll) complex of CPZ7 but this action noticeably exceeded that of CPZ (Figure 2d). It was interesting that some of metal complexes were effective; however, the biological action did not greatly exceeded the mdr reversal action of the parent compounds.

The measurements of thermal stability of DNA showed some interaction between the phenothiazine metal complexes and DNA. The increased melting temperatures of DNA in the presence of metal complexes indicates an interaction with DNA and stabilisation of the helix. CPZ and TFP can be seen to stabilise the DNA helix by intercalation causing a slight increase in the thermal denaturation temperature. A similar effect was seen with the TFP-Sn(IV) the CPZ-Sn(IV) and the TFP-Cu(ll) coordination complexes indicating an interaction with the DNA helix whilst metal ions alone showed no significant stabilisation of the helix. The chlorides of Pt, Pd and V degraded the DNA resulting in a linear thermal stability profile. The coordination complexes of these metals with CPZ and TFP however decreased the thermal stability of DNA. The phenothiazines appear to be exerting a protective effect, protecting the DNA against total degradation caused by the metal ions alone (Table III).

Table III. *Change in Thermal Denaturation Temperature (T) of CT DNA in the presence of phenothiazines and metal complexes.*

Compounds	DNA: comp. = 10:1	ΔTm	DNA: comp. = 5:1	ΔTm	DNA: comp. = 2.5:1	ΔTm
CPZ		0.2		0.44		0.67
TFP		0.91		1.22		1.6
CPZ-Pt(II)				-2.7		
Pt(II)				*		
CPZ-Sn(IV)		1.2		1.4		1.34
Sn(IV)		0.2		0.1		0.23
TFP-Cu(II)		0.93		1.4		1.54
Cu(II)		0.70		-0.14		0.54
TFP-Ni(II)		2.38		0.93		2.3
Ni(II)		0.1		0.3		0.34
TFP-Pd(II)				-5.4		
Pd(II)				*		
TFP-V(IV)		-2.3		-10.1		
V(IV)				*		
TFP-Sn(IV)		2.0		1.0		1.4
Sn(IV)		0.2		1.0		0.23

* Resulted in degradation of the DNA

Discussion

Apparently the phenothiazines and their metal co-ordination complexes were able to reduce the SOS function in bacteria (reducing the bacteriocin induction) and tumour cells (reducing the ABC transporter activity). It has suggested, that the inhibition of the two different SOS function is due to the complex formation between the phenothiazines and the inducible SOS gene or some regulator proteins. Based on this working hypothesis, the complex formation with native B form DNA was studied by measuring thermal stability of DNA.

The results suggest that the co-ordinating metals alter the fine chemical structure (superdelocalizibility of π-electrons) and biological action of phenothiazines.

The CPZ-V(IV) complex has been shown to be a more effective inhibitor of the SOS function in the cultures of *B. megaterium* than TFP and it had a reversal action on mdr of tumour cells. The T_m values suggest that an explanation for this action may be an interaction between the DNA and the phenothiazine-metal co-ordination complexes.

Metal ions react with a variety of electron donor sites on polynucleotides. There are three main sites of interaction, the phosphate moieties of the ribose-phosphate backbone, the electron donor groups of the basis and the sugar part of the polynucleotides. The fourth but still significant interaction could occur by the intercalation of metals or metal complexes and altering the hydrogen-bonding network. Interaction with ribose moieties under such experimental conditions is neglible. Reactions with the phosphate means stabilisation of ordered structure or the cleavage of phoshodiester bonds at high temperature. Although the binding is not specific, the

result is the neutralisation of the array of negative charges on the double helix thus stabilising it. This stabilisation is accompanied by an increase in the melting temperature of DNA. Concerning the metal ions, the preference for phosphate over the base association decreases in the order Mg(II) > Co(II) > In(II) > Mn(II) > Zn(II) > Cu(II) (15). Reaction with bases means destabilisation of ordered structures, since metal ions or complexes can bind to the base stacking interactions that hold together the two strands of DNA. This interaction is accompanied by a decrease in the melting temperature.

It may be suggested from the thermal denaturation studies (Table III) that the SOS inhibitory function and the reversal action on mdr of tumour cells is due to the denaturing effect of Pd, Pt or V ions on the cellular DNA. Co-ordination of these ions with CPZ or TFP provides a means by which these ions may be selectively carried to the DNA helix, as the phenothiazines are known to interact with the DNA helix (16). At the helix the Pt, Pd and V ions act to destabilise the helix, as evidenced by the observed decrease in the thermal stability of the DNA. A possible mechanism for this might be by the Pt, Pd or V ions interfering with hydrogen bonding between the nitrogenous base pairs, or the metal complexes interact with bases and destabilised the ordered structures.

The inhibitory effect on megacine induction and on the mdr can be improved by complex formation of phenothiazines with various metal ions. Therefore, we can conclude, that the specific action of phenothiazine ring system can be basically modified by multivalent co-ordinating metal ions.

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References

1 Lum BL, Gosland MP, Kaubisch S and Sikic BI: Molecular targets in oncology: implications of the multidrug resistance gene. Pharmacotherapy *13(2):* 88-109, 1993.

2 Asakuno K, Kohno K, Uchiumi T, Kubo T, Sato S, Isono M and Kuwano-M: Involvement of a DNA binding protein, MDR-NF1/YB-1, in human MDR1 gene expression by actinomycin-D. Biochem Biophys Res Commun I *99(3):* 1428-1435, 1994.

3 Venetianer A, Pirity M and Hever-Szabo A: The function of heat-shock proteins in stress tolerance. Cell Biol Int *18(6):* 605-615, 1994.

4 Walther W. Wendt J and Stein U: Employment of the mdr1 promoter for the chemotherapy- inducible expression of therapeutic genes in cancer gene therapy. Gene Ther *4(6):* 544-552, 1997.

5 Barancik M, Polekova L, Mrazova T, Breier A, Stankovicova T and Slezak J: Reversal effects of several Ca(2+)-entry blockers, neuroleptics and local anaesthetics on P-glycoprotein- mediated vincristine resistance of L 1210/VCR mouse leukaemic cell line. Drugs Exp Clin Res *20(1):* 13-18, 1994

6 Ayesh S Shao YM and Stein WD: Co-operative, competitive and non competitive interactions between modulators of P-glycoprotein. Biochim Biophys Acta *1316(1):* 8-18, 1996.

7 Nagy L, Korecz L, Kiricsi I, Zsikla L and Burger K: Synthesis, Mossbauer, and IR Spectroscopic Studies and Thermal Behavior of Diorganotin(IV) Complexes with Carbohydrate Ligands. Struct Chem *2:* 231-2380, 1991.

8 Holland IB and Roberts CF: Some Properties of a New Bacteriocin-285 Formed by Bacillus megaterium. J Gen Microbiol *35:* 271-285, 1964.

9 Ivanovics G, Nagy E and Alföldi L: Megacinogeny: inducible synthesis of a new immunospecific substance. Acta Microbiol Acad Sci Hung *6:* 161-169, 1959.

10 Rad-niknam M and Sharples D: Potential antitumor agents. Synthesis, DNA bonding studies and biological activity of 9-aminoacridinyl derivatives of citrulline arginine. Arzneim.-Forsch./Drug Res *40:* 287-289, 1990.

11 Angerer LM and Moudrianakis EN: Interaction of ethidium bromide with whole and selectively deproteinized deoxynucleoproteins from calf thymus. J Mol Biol *63:* 505-521, 1972.

12 Wells RD, Larson JE, Grant RC, Shortle BE and Cantor CR: Physicochemical studies on polydeoxyribonucleotides containing defined repeating nucleotide sequences. J Mol Biol *54:* 465-497, 1970.

13 Ebrahimi SES, Parkinson JA, Fox KR, McKie JH, Barber J and Douglas KT: Studies of the interaction of a meta-hydroxy analogue of Hoechst 33258 with DNA by melting temperature, footprinting and high resolution ^{1}H NMR spectroscopy. J. Chem Soc Chem Commun 1398-1400, 1992.

14 Ebrahimi SES, Bibby MC, Fox KR and Douglas KT: Synthesis DNA-binding, footprinting and *in vitro* antitumor studies of a meta-hydroxy analogue of Hoechst 33258. Anti-Cancer Drug Design *11:* 463-479, 1995.

15 Eichhorn GL and Shin YA: Interaction of Metal Ions with Polynucleotides and Related Compounds. XII. The relative Effect of Various Metal Ions on DNA Helicity. J Am Chem Soc *90:* 7323-7328, 1968.

16 Barabas K and Molnar J: Lack of the correlation between intercalation and plasmid curing ability of some tricyclic compounds. Acta Microbiol Acad Sci Hung *27:* 55-61, 1980.

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VII.

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CANCER LETTERS

Anti-psychotic drugs reverse multidrug resistance of tumor cell lines and human AML cells ex-vivo

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Abstract

Anti-psychotic drugs are used in cancer patients undergoing chemotherapy frequently and the concomitantly used drugs may alter the pharmacokinetics of each other. One reason for the alteration of pharmacokinetics may be the modulation of the function of P-glycoprotein, whose efflux pump occurs in resistant cancer cells, in human intestine and in the blood–brain barrier. For this reason we tested the effect of several anti-psychotic drugs on the multidrug-resistant pump, P-glycoprotein. We found that in the MDR gene transfected L121C MDR, L5178 MDR and in the KB-V-1 cells selected for resistance some anti-psychotic drugs block the function of P-glycoprotein. Blood cells of two treatment-resistant leukemic patients also showed increased uptake of daunorubicin if treated ex vivo with the anti-psychotic drugs. Our results suggest that pharmacokinetic studies should be performed prior to concomitant clinical use of such drugs which block P-glycoprotein function.

Keywords: Reversal; Multidrug resistance; Amitriptyline; Fluphenazine; Maprotiline; Trimipramine; Desipramine; Imipramine; Haloperidol; Cyclosporin A; In vitro; In vivo; In patients

1. Introduction

Resistance to cytotoxic drugs is a major problem in cancer chemotherapy [1]. Different mechanisms are involved in cytotoxic drug resistance. One of the most important mechanisms is the P-glycoprotein (P-gp) mediated multidrug resistance [2]. P-gp is responsible for accelerated efflux of chemically unrelated drugs from cancer cells. Interference with the function of P-gp, can promote effectiveness of cancer chemotherapeutic drugs. Several compounds are in clinical trials for modulation of P-gp related resistance in cancer [3].

It is not uncommon to use anti-psychotic drugs in cancer patients [4–6]. The concomitantly used cancer chemotherapeutic and anti-psychotic drugs may alter each other's pharmacokinetics in cancer patients. If the anti-psychotic drug also effects P-gp function then the pharmacokinetics of the anticancer drugs may change in cancer cells and also through the blood–brain barrier [7].

Tricyclic anti-psychotic drugs were shown to exert a variety of biological effects on the subcellular and cellular levels. For example, chlorpromazine (CPZ),

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trifluoperazine and clozapine inhibit certain proteases, acetylcholine esterase and affect Ca^{2+} metabolism [8]. Fluphenazine, a phenothiazine type anti-psychotic drug, some of its analogues [9], butaclamols stereoisomers [10] and the chemically related thioxanthene-type compounds [11] were shown to inhibit P-gp function in some MDR1 gene expressing cell lines. Most of these tricyclic and the other anti-depressive drug molecules are lipophilic and possess a positive charge due to their nitrogen atom. These chemical characteristics were shown to be important for agents that affect the function of P-gp [1,12]. Prochlorperazine, the tricyclic anti-emetic drug was shown to be effective as a resistance modifier in phase I clinical studies [13].

For the above reasons we have studied the effect of some anti-psychotic drugs on the function of P-gp. In our study, we used the transfected leukemia cell lines L1210 MDR, L5178 MDR and the human adenocarcinoma cell line KB-V-1 selected for resistance and peripheral blood lymphocytes obtained from leukemic patients.

2. Materials and methods

2.1. Cells

Peripheral human blood (PBL) samples were obtained from volunteer cancer patients and PBL was prepared by Ficoll–Hypaque density gradient centrifugation as described earlier [14]. The sensitive leukemia cell lines, L1210, L5178 and its MDR1 gene transfected resistant pair were obtained from Dr. Gottesman of NIH, and were maintained as described earlier [15]. MDR expressing cells were cultured in the presence of colchicine up to 48 h before being used in the drug uptake assay. The sensitive KB-3-1 and the resistant KB-V-1 cells were cultured as described previously [16].

2.2. Flow cytometric drug uptake assay

The flow cytometric drug uptake assay was carried out essentially as described earlier [17]. In brief, fluorescent substrate, Rhodamine 123 (R123) (0.08 μM), or daunorubicin (DR) (1 μM) uptake was assessed after 30 min incubation time at 37°C. Anti-psychotic drugs were added to cell suspensions at room temperature 10 min before the fluorescent substrate, 10^6 cells/ml, in glucose containing PBS.

Fluorescence histograms were obtained of 10^4 cells with a Becton Dickinson FACScan (Mountain View, CA) or with a FACStar Plus instrument (Becton Dickinson). The laser was tuned to 488 nm and fluorescence was detected at 525 nm (FL-1 channel) or at 585 nm for R123 and daunorubicin (DR), respectively. Mean fluorescence intensities were calculated by the C30 software of the instrument. Results represent one typical of two or three experiments performed with new cell preparations.

2.3. Chemicals

All anti-psychotic drugs, Rhodamine 123 (R123), Cyclosporin A (CsA) and daunorubicin were obtained from Sigma (St. Louis, MO).

2.4. Patients

Patients were consent blood donors at the University Medical School of Debrecen. Patient SzL was diagnosed as having acute myeloid leukemia (AML). The first regiment of treatment was with DR (45 mg/m^2 for 3 days) and Alexan (100 mg/m^2 for 7 days). After relapse, the second regiment of treatment was TAD/COAP (Tioguanin, Alexan, Daunorubicin/Cyclophosphamide, Vincristine, Alexan, Prednisolone). At the time of the blood sample the WBC was 28.5×10^9 cells/l and the blast count was 38% as measured by Giemsa staining. This patient was in complete relapse at the time of the flow cytometric analysis and later became diseased. Patient PL was diagnosed with AML. The first regiment of treatment was adriamycin (AM), (45 mg/m^2 for 3 days) and Alexan (10 mg/m^2 for 7 days). After relapse, the second regiment was TAD/COAP. At the time of the blood sample the WBC was 2.2×10^9 cells/l and blasts were not detected. At the present time the patient is asymptomatic. Patient NV was diagnosed with AML. The first regiment of treatment was DR (45 mg/m^2 for 3 days) and Alexan (100 mg/m^2 for 7 days). This treatment was repeated after objective improvement. At the time of the blood sample WBC was 7.1×10^9 cells/l and the blast count was 9.8%. This patient later died.

Table 1
Influence of anti-psychotic drugs on the uptake of Rhodamine 123, the P-gp substrate, into L1210, L5178 cells and the MDR derivatives, KB-3-1 and KB-V-1 (MDR) cells[a]

Anti-psychotic drugs	Conc. (μg/ml)	Relative fluorescence intensity					
		Cells					
		L1210	L1210 MDR	L5178	L5178 MDR	KB-3-1	KB-V-1
Amitriptyline	0.5	1.19	3.02	0.62	3.27	–[b]	–
	1	–	–	0.67	3.50	–	1.96
	5	1.19	5.87	0.54	4.81	0.86	5.98
Fluphenazine	0.5	1.49	4.02	0.75	4.45	–	–
	1	–	–	0.76	7.44	–	1.37
	5	1.16	8.45	0.68	13.30	0.73	2.89
Maprotiline	0.5	1.13	2.54	0.68	3.22	–	–
	1	–	–	0.60	3.08	–	–
	5	1.02	4.63	0.50	8.05	0.71	1.68
Trimipramine	1	1.22	2.91	–	–	–	1.37
	5	1.15	6.30	–	–	0.81	2.05
Desipramine	1	–	1.73	–	–	–	1.37
	5	–	2.80	–	–	0.78	1.55
Imipramine	0.5	–	1.28	0.82	3.04	–	–
	1	–	–	0.74	3.45	–	–
	5	–	2.82	0.70	4.80	–	1.98
Haloperidol	0.5	1.03	5.39	0.71	2.28	–	–
	1	–	–	0.64	2.04	–	1.99
	5	1.15	13.20	0.51	2.11	0.89	3.37
Doxepin	0.5	–	1.19	0.96	2.40	–	–
	1	–	–	0.81	2.67	–	–
	5	–	2.95	0.57	4.48	–	–
Cyclosporin A	5	1.21	7.05	0.85	12.08	0.90	4.51

[a] Fluorescence intensities are ratios of means of histograms obtained with and without drugs. Each histogram was obtained from 10^4 cells using flow cytometry as described in Section 2. Typical results of three independent experiments. Rhodamine 123 concentration was 5.2 μM and that of daunorubicin was 3 μM.

[b] –, not tested.

3. Results and discussion

Previous reports by Ford et al. [9,18] and structural features of several anti-psychotic drugs prompted us to test them as blockers of P-gp, the multidrug efflux pump. Initial testing of the anti-psychotic drugs was done in L1210, L5178 parental and L1210 MDR, L5178 MDR cells and with the substrate R123. Table 1 shows relative mean fluorescence intensities of histograms obtained in the absence and the presence of 0.5, 1 or 5 μg/ml of the drugs. Treatment of L1210 MDR cells with CsA (5 μg/ml), a known P-gp blocker, increased the relative fluorescence intensity about 7-fold in the L1210 MDR cells. All tested anti-psychotic drugs increased the relative fluorescence intensity, uptake of R123, into L1210 MDR cells, haloperidol and fluphenazine being the most active. In the L5178 MDR cell line fluphenazine and maprotiline were the most effective compounds. None of the tested drugs significantly changed R123 uptake into the parental cells. Similar results were obtained with KB cells using DR as fluorescent substrate (Table 1).

Again, relative fluorescence intensities are ratios of mean fluorescence of histograms obtained of drug treated and untreated cell populations. In these experiments DR was used as a fluorescent substrate of P-gp since it was also used as a chemotherapeutic agent in the patients. The anti-psychotic drugs increased the relative fluorescence of KB-V-1 but not of the KB-

Table 2
Influence of anti-psychotic drugs on the uptake of daunorubicin, as P-gp substrate, into PBL of patients[a]

Anti-psychotic drugs	Conc. (μg/ml)	Relative fluorescence intensity		
		Patients		
		SzL	PL	NV
Amitriptyline	1	–[b]	–	–
	5	1.48	1.39	1.10
Fluphenazine	1	–	–	–
	5	1.24	1.53	0.90
Maprotiline	1	–	–	–
	5	1.86	1.32	1.03
Trimipramine	1	–	–	–
	5	1.53		
Desipramine	1	–	–	0.90
	5	1.65	–	–
Imipramine	1	–	–	–
	5	1.42	1.00	1.00
Haloperidol	1	–	–	–
	5	1.44	1.37	0.98
Cyclosporin A	5	1.39	1.32	0.90

[a] Results are expressed as in the legend to Table 1. A description of the patients (SzL, PL and NV) is given in Section 2. Typical results of two independent experiments.
[b] –, not tested.

3-1, parental cells. The increase was close to that with CsA, the known P-gp blocker, for amitriptyline, fluphenazine and haloperidol. However, efflux of DR from KB-V-1 cells was not blocked to the same extent as in the L1210 MDR and L5178 MDR cells, relative to CsA (Table 1). We attribute this lack of correlation between the three cell lines to the fact that we used different substrates, R123 and DR, with the L1210, L5178 and the KB cells, respectively, which can contribute to differential sensitization with CsA. In addition, a differential effect of the anti-psychotic drugs on the membranes of these three cell lines is possible. It was shown previously that some P-gp blockers may exert their effects indirectly through the plasma membrane without being a substrate [15].

Because of the above-demonstrated blocking effect of the tested anti-psychotic drugs on P-gp, we tested these drugs in peripheral blood lymphocytes (PBL) of drug treatment resistant patients using DR as a fluorescent substrate. Table 2 shows the uptake of DR into PBL of patients.

Relative to L1210 MDR, L5178MDR and to KB-V-1 cells, the known P-gp blocker, CsA had a small effect on DR efflux in the PBL of the patients SzL and PL. However, most of the tested anti-psychotic drugs had a comparable effect to CsA in PBL of both of these patients (Table 2).

Cells from AML patient NV, although resistant to treatment in the clinic, could not be sensitized by CsA or with the anti-psychotic drugs for uptake of DR (Table 2). The reason for the difference among the three PBL for sensitization could not be established, for example, by mAb staining. Future investigations are planned to include more detailed characterization of clinically resistant AML cells, which do not respond to P-gp blockers.

Anti-psychotic drugs are used at a blood level of 10 to 500 ng/ml, depending on the drug used and on the sensitivity of the patient [11,19]. A double concentration of the highest clinical blood level, 1 μg/ml, showed small but significant blocking of P-gp in L1210 MDR, L5178 MDR and KB-V-1 cells (Table 1). PBL of the patients could not be tested at the 1 μg/ml dose level, due to an insufficient amount of blood samples. However, based on results with CsA at a comparable dose level (5 μg/ml) it is expected that uptake of DR into PBL would be enhanced by 1 and 0.5 μg/ml of some of the anti-psychotic drugs.

Taken together, using the model cell lines L1210 MDR, L5178 MDR and KB-V-1 and the fluorescent indicator R123 and DR, we could show that the clinically used anti-psychotic drugs may significantly modulate the uptake of substrates of P-gp into MDR cells. We have also tested this possibility in a pilot study, using PBL prepared from blood of drug-resistant leukemic patients. Resistance was based on clinical experience and was verified on PBL by the effect of CsA on the uptake of DR. In these PBL cells we compared the effects of the anti-psychotic drugs with that of CsA for the uptake of DR, the drug used for the treatment of two of the patients. The ex vivo results indicate that treatment with some of the anti-psychotic drugs result in as much change in DR uptake as CsA does into the PBL of two out of three patients. Our results point in the direction of necessary pharmacological studies when anti-psychotic drugs and cancer chemotherapeutic agents are used in combination.

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References

[1] M.M. Gottesman, I. Pastan, Biochemistry of multidrug resistance mediated by the multidrug transporter, Annu. Rev. Biochem. 62 (1993) 385–427.

[2] H.D. Preisler, Multidrug resistance is more than *MDR1* activity, Leukemia Res. 19(7) (1995) 429–431.

[3] M. Dicato, C. Duhem, M. Pauly, F. Ries, Multidrug resistance: molecular and clinical aspects, Cytokines Cell. Mol. Ther. 3 (1997) 91–100.

[4] R.M. Cole, F. Robinson, L. Harvey, K. Trethowan, V. Murdoch, Successful control of intractable nausea and vomiting requiring combined ondansetron and haloperidol in a patient with advanced cancer, J. Pain Symptom Manage. 9 (1994) 48–50.

[5] F. Stiefel, J. Holland, Delirium in cancer patients, Int. Psychogeriatr. 3 (1991) 333–336.

[6] C.J. Vecht, A.M. Hoff, P.J. Kansen, M.F. de-Boer, D.A. Bosch, Types and causes of pain in cancer of the head and neck, Cancer 70 (1992) 178–184.

[7] A.H. Schinkel, E. Wagenaar, C.A.A.M. Mol, L. van Deemter, P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs, J. Clin. Invest. 97(11) (1996) 2517–2524.

[8] J. Molnár, I. Sohár, J. Kovács, Z. Rakonczay, H. Rausch, Effect of tricyclic compounds on membrane binding of bivalent cations, activities of acetylcholinesterase and some tissue proteases, In Vivo 7 (1993) 431–434.

[9] J.M. Ford, W.C. Prozialeck, W.N. Hait, Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance, Mol. Pharmacol. 35 (1989) 105–115.

[10] D. Szabó, J. Molnár, The role of stereoselectivity of chemosensitizers in the reversal of multidrug resistance of mouse lymphoma cells, Anticancer Res. 18 (1998) 3039–3044.

[11] A.H. Glassman, J.M. Perel, Tricyclic blood levels and clinical outcome: a review of the art, in: A.M. Lipton, A. DiMascic, K.F. Killam (Eds.), Psychopharmacology, a Generation of Progress, Raven, New York, 1978, pp. 917–921.

[12] J.S. Lee, K. Paull, M. Alvarez, C. Hose, A. Monks, M. Grever, A.T. Fojo, S.E. Bates, Rhodamin efflux patterns predict P-glycoprotein substrates in the national cancer institute drug screen, Mol. Pharmacol. 46 (1994) 627–638.

[13] K.S. Sridhar, A. Krishan, T.S.A. Samy, R.C. Duncan, A. Sauerteig, G.V. McPhee, M.E. Auguste, P.W. Benedetto, Phase I and pharmacokinetics studies of prochlorperazine 2-h i.v. infusion as a doxorubicin-efflux blocker, Cancer Chemother. Pharmacol. 34 (1994) 377–384.

[14] A. Aszalos, P.S. Pine, J.L. Weaver, P.E. Rao, Cytochalasin D modulates CD4 crosslinking sensitive mitogenic signal in T lymphocytes, Cell. Immunol. 157 (1994) 81–91.

[15] A. Aszalos, P.S. Pine, R. Pandey, M.M. Gottesman, Behavior of N-acylated daunorubicins in MDR1 gene transfected and parental cells, Biochem. Pharmacol. 50 (1995) 889–892.

[16] J.L. Weaver, P.S. Pine, A. Aszalos, P.V. Schoenlein, S.J. Currier, R. Padmanabhan, M.M. Gottesman, Laser scanning and confocal microscopy of daunorubicin, doxorubicin and rhodamin 123 in multidrug-resistant cells, Exp. Cell Res. 196 (1991) 323–329.

[17] J.L. Weaver, G. Szabó Jr., P.S. Pine, M.M. Gottesman, S. Goldenberg, A. Aszalos, The effect of ion channel blockers, immunosuppressive agents and other drugs on the activity of the multidrug transporter, Int. J. Cancer 54 (1993) 456–461.

[18] J.M. Ford, E.P. Bruggemann, I. Pastan, M.M. Gottesman, W.N. Hait, Cellular and biochemical characterization of thioxanthenes for reversal of multidrug resistance in human and murine cell lines, Cancer Res. 50 (1991) 1748–1756.

[19] R.J. Baldessarini, B.M. Cohen, M.H. Teicher, Significance of neuroleptic dose and plasma level in the pharmacologic treatment of psychoses, Arch. Gen. Psychiatry 45 (1988) 79–91.

VIII.

ANTICANCER RESEARCH *19:* 1859-1864 (1999)

Chemical Structure and Tumor Type Specificity of "Half-Mustard Type" Phenothiazines

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Abstract. *The antiproliferative activity of six "half-mustard type" phenothiazines against a total of 54 tumor cell lines: 4 leukemia, 9 non-small-cell lung, 7 colon-, 5 CNS-, 8 melanoma, 6 ovarian-, 8 renal-, 1 prostate and 6 breast cancer was determined by NCI-Information Intensive-Approach. The C-2 position of phenothiazines were substituted with H, Cl and CF_3 groups. The half-mustard and ring system was linked either by a propylene or a butylene bridge. Colon-cancer cell showed the highest sensitivity against "half-mustard type" phenothiazines, followed by leukemia, melanoma, prostate-, CNS-, breast-, lung-, renal and ovarian cancer cells. These data suggest the "cancer-type-specific" antitumor action of "half-mustard type" phenothiazines.*

Phenothiazines interact with various macromolecules and cells (1-6). Some of them prevent chemical carcinogenesis (7), due to their antimutagenic (8) or differentiation-inducing action (9), synergise the antitumor activity of 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) and hyperthermia (6, 10), and show the radiosensitizing effect (11). BCNU enhanced the antiproliferative action of the phenothiazines (11-15).

Phenothiazines exert antitumor activity *in vitro* and *in vivo* (1, 12, 13, 16). Introduction of different substituents into phenothiazine skeleton might alter their antitumor (1, 12-14) and antiretroviral (15, 17) activity. Based on previous studies, more potent "half-mustard type" phenothiazines with alkylamino side linkers were synthetized (16). In the preliminary experiments, the phthalimidophenothiazines [**1-6**] were found to be inactive (Figure 1) (18). However, a new information-intensive study of NCI (19) has displayed the antiproliferative activity of the alkylurea derivatives [**7-12**] against 54 cell lines (nine cancer cell types). The "half-mustard type" phenothiazines with butylene linker were active against leukemia, non-small cell lung cancer and colon cancer, but ineffective against renal and ovarian cancer cells. The structure-activity relationship studies has shown that the sensitivity of AIDS-related leukemia and lymphoma is different from (20) that of other types of cancer cell lines (18). The "half-mustard type" phenothiazines with butylene linker were more effective than those with propylene linker. Therefore, the role of multidrug resistance efflux pump and cancer type specific antiproliferative action of "half-mustard type" phenothiazines were promising to be analyzed in more details. Therefore, we investigated here the antitumor activity of six "half-mustard type" phenothiazines against various cancer cell lines to design more effective anticancer drugs.

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Key Words: "Half-mustard type" phenothiazines, structure, antitumor activity, specificity.

Materials and Methods

Chemicals. All related phenothiazines [**1-12**] were synthesized as previously described (16) (Figure 1).

Assay for antitumor activity. The 50% growth inhibitory concentration (GI50), 100% tumor growth inhibitory concentration (TGI) and 50% lethal concentration (LC50) values of six 1-(2-chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkylureas [**7-12**] were determined by the dose-response curve, using 4 leukemia, 9 non-small cell lung carcinoma, 7 colon cancer, 5 CNS-cancer, 8 melanoma, 6 ovarian cancer, 8 renal cancer, 1 prostate cancer and 6 breast cancer cell lines as target cells. The mean values of GI50, TGI and LC50 were calculated in each tumor group for the six tested compounds [**7-12**]. The $\log_{10}$ values were converted to molar concentration ($\times 10^{-5}$ M) using the previous data (18) for compounds [**7-12**] (Table Ia), and compared to each other. The compounds acting at the lowest concentrations were considered to be the most effective ones on the particular tumor cell types. The GI50 and TGI were considered as values corresponding mainly to cytostatic effects, while the LC50 values were thought to represent the cell killing capacities of the "half-mustard type" phenothiazines.

Compound	R	n	NSC No.[1]	ST No.[2]
10-[N-(Phthalimido)alkyl]-2-substituted-10H-phenothiazine				
1	H	3	D681647	ST50000
2	H	4	D681648	ST50001
3	Cl	3	D681649	ST50002
4	Cl	4	D681650	ST50003
5	CF_3	3	D681651	ST50004
6	CF_3	4	D681652	ST50005
1-(2-Chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylurea				
7	H	3	D681653	ST50006
8	H	4	D681654	ST50007
9	Cl	3	D681655	ST50008
10	Cl	4	D681656	ST50009
11	CF_3	3	D681657	ST50010
12	CF_3	4	D681658	ST50011

Figure 1. *Structures of 10-[N-(phthalimido)alkyl-2-substituted-10H-phenothiazines* ***[1-6]****, and 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas* ***[7-12]****. 1) NCI code number. 2) SCRIPTGEN code number.*

Table Ia. *Anticancer activity of six 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas* ***[7-12]*** *against nine different groups of cancer lines.*

Compd	Anticancer activity								
	Leukemia (4)	Non-small cell lung (9)	Colon-cancer (7)	CNS-cancer (5)	Melanoma (8)	Ovarian cancer (6)	Renal cancer (8)	Prostate cancer (1)	Breast-cancer (6)
					$\log_{10}$ (GI50)				
7	-4.98	-4.84	-4.98	-4.86	-4.88	-4.86	-4.96	-4.97	-4.84
8	-5.32	-5.12	-5.40	-5.06	-5.23	-4.99	-5.13	-5.27	-5.23
9	-5.73	-5.40	-5.69	-5.59	-5.70	-5.25	-5.33	-5.60	-5.44
10	-5.81	-5.60	-5.85	-5.71	-5.78	-5.51	-5.56	-5.68	-5.68
11	-5.72	-5.45	-5.69	-5.84	-5.61	-5.21	-5.31	-5.59	-5.58
12	-5.80	-5.53	-5.78	-5.70	-5.74	-5.41	-5.53	-5.61	-5.58
					$\log_{10}$ (TGI)				
7	-4.54	-4.55	-4.62	-4.57	-4.58	-4.57	-4.97	-4.65	-4.56
8	-4.75	-4.68	-4.95	-4.66	-4.76	-4.62	-4.70	-4.74	-4.76
9	-5.34	-4.97	-5.33	-5.10	-5.40	-4.74	-4.89	-4.92	-5.07
10	-5.38	-5.19	-5.55	-5.28	-5.49	-4.93	-5.15	-5.09	-5.30
11	-5.28	-5.01	-5.32	-5.07	-5.25	-4.72	-4.86	-4.87	-5.09
12	-5.37	-5.10	-5.44	-5.23	-5.48	-4.83	-5.02	-4.92	-5.15
					$\log_{10}$ (LC50)				
7	-4.12	-4.26	-4.30	-4.27	-4.28	-4.27	-4.29	-4.33	-4.25
8	-4.23	-4.31	-4.56	-4.31	-4.39	-4.29	-4.34	-4.37	-4.32
9	-4.85	-4.55	-4.99	-4.61	-5.04	-4.34	-4.49	-4.45	-4.57
10	-4.68	-4.69	-5.24	-4.73	-5.22	-4.37	-4.69	-4.52	-4.86
11	-4.39	-4.58	-4.94	-4.51	-4.84	-4.31	-4.47	-4.42	-4.62
12	-4.86	-4.60	-5.08	-4.70	-5.16	-4.29	-4.50	-4.44	-4.68

Table Ib. *Anticancer effects of six 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas [7-12] on nine different groups of cancer lines.*[a]

Compd	Anticancer activity								
	Leukemia (4)	Non-small cell lung (9)	Colon-cancer (7)	CNS-cancer (5)	Melanoma (8)	Ovarian cancer (6)	Renal cancer (8)	Prostate cancer (1)	Breast-cancer (6)
					GI50 (x 10^{-5} M)				
7	1.06	1.44	1.06	1.37	1.32	1.39	1.11	1.07	1.46
8	0.48	0.76	0.40	0.87	0.59	1.02	0.74	0.54	0.60
9	0.19	0.40	0.20	0.26	0.20	0.56	0.47	0.25	0.37
10	0.16	0.25	0.14	0.20	0.17	0.31	0.28	0.21	0.21
11	0.19	0.35	0.21	0.14	0.24	0.62	0.49	0.26	0.26
12	0.16	0.29	0.17	0.20	0.18	0.39	0.29	0.25	0.26
					TGI (x 10^{-5} M)				
7	2.91	2.81	2.39	2.72	2.64	2.72	1.07	2.24	2.77
8	1.77	2.11	1.13	2.21	1.76	2.39	2.02	1.82	1.74
9	0.45	1.07	0.46	0.80	0.40	1.81	1.30	1.20	0.85
10	0.42	0.64	0.28	0.52	0.32	1.18	0.71	0.81	0.50
11	0.52	0.97	0.48	0.85	0.57	1.93	1.37	1.35	0.81
12	0.43	0.79	0.36	0.59	0.33	1.50	0.95	1.20	0.71
					LC50 (x 10^{-5} M)				
7	7.59	5.52	5.03	5.37	5.22	5.33	5.11	4.68	5.68
8	5.96	4.85	2.75	4.92	4.10	5.15	4.60	4.27	4.83
9	1.41	2.83	1.03	2.47	0.92	4.54	3.23	3.55	2.68
10	2.11	2.04	0.57	1.86	0.60	4.25	2.07	3.02	1.39
11	4.04	2.62	1.14	3.09	1.45	4.88	3.42	3.80	2.40
12	1.40	2.53	0.83	1.99	0.69	5.11	3.17	3.63	2.08

[a] Transformation: $10^{Ia} \times 10^{5} = Ib$

Results and Discussion

For improving the antitumor effects of phenothiazines, we have newly synthesized "half-mustard type" phenothiazines by modifing the alkylamino side chains and investigated their antitumor activity against a total of 54 tumor cell lines (18) such as leukemia, melanoma, lung, colon, ovarian, breast, renal and prostate cancer, and brain tumor cell lines.

The present study shows that the substituent at position 2 in the phenothiazine ring and the number of linker are important factors for antitumor activity. Obviously, the configuration of the lone pair localized in the ring nitrogen (N) of phenothiazine and the length between the phenothiazine ring nitrogen (N) and terminal nitrogen (N) of methylene group might play a role in the antitumor activity of the new compounds. The compounds with butylene bridge yielded more potent compounds than those with the propylene bridge. The active domain of the derivatives might attack the cytosine or more likely guanosine residues in DNA by electrophilic covalent binding or by polarizability of compounds. On the basis of computer aided studies, some correlations were found between the biological activities and values of dipole moments and entropy of the newly synthetized phenothiazine alkylureas (8a). Additionally, the

Table IIa. *Sensitivity of nine types of cancer cells against five 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas [7-12].*

Cancer cell type	GI50 (x 10^{-5} M)	Cancer cell type	TGI (x 10^{-5} M)	Cancer cell type	LC50 (x 10^{-5} M)
colon cancer	0.36	colon cancer	0.85	colon cancer	1.86
leukemia	0.37	melanoma	1.00	melanoma	2.16
prostate cancer	0.43	leukemia	1.08	breast-cancer	3.18
melanoma	0.45	breast-cancer	1.23	CNS-cancer	3.28
CNS-cancer	0.51	renal cancer	1.23	non-small cell lung	3.40
breast-cancer	0.53	CNS-cancer	1.28	renal cancer	3.60
renal cancer	0.56	non-small cell lung	1.40	leukemia	3.75
non-small cell lung	0.58	prostate cancer	1.44	prostate cancer	3.82
ovarian cancer	0.71	ovarian cancer	1.92	ovarian cancer	4.88

Table IIb. *Sensitivity of nine types of cancer cells against five 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas [8-12].*

Cancer cell type	GI50 (x 10^{-5} M)	Cancer cell type	TGI (x 10^{-5} M)	Cancer cell type	LC50 (x 10^{-5} M)
colon-cancer	0.22	colon-cancer	0.54	colon-cancer	1.27
leukemia	0.23	melanoma	0.68	melanoma	1.55
melanoma	0.28	leukemia	0.72	breast-cancer	2.68
prostate cancer	0.30	breast-cancer	0.92	CNS-cancer	2.86
CNS-cancer	0.33	CNS-cancer	0.99	non-small cell lung	2.97
breast-cancer	0.34	non-small cell lung	1.12	leukemia	2.98
non-small cell lung	0.41	renal cancer	1.27	renal cancer	3.30
renal cancer	0.45	prostate cancer	1.28	prostate cancer	3.65
ovarian cancer	0.58	ovarian cancer	1.76	ovarian cancer	4.78

1) The values of compound [7] are omitted.

urea portion of "half-mustard type" phenothiazines have shown significant variability in the energy (eV) of lone pair orbital (n) and a lone pair electron density (ϱ^n) in n orbital of N1, O, and N3 atoms (data not shown) (8b) (Figure 2).

When the $\log_{10}$ values of GI50, TGI and LC50 of 6 compounds [7-12] against 54 different cancer cell lines were compared, an interesting correlation was found (Table Ia). Then, the logarithmic values were converted to molar concentrations (Table Ib). The mean value of GI50, TGI and LC50 concentrations of compounds [7-12] was calculated at first. Then, the nine main tumor groups were ranked from the lowest values (more sensitive) of GI50, TGI and LC50 towards the highest values (more resistant) (Table IIa). Secondly, compound [7] was omitted from Table IIb, because of its extremely low antitumor activity probably due to the permeability barrier, as compared to other five compounds [8-12]. The compound [7] has the lowest ground state dipole moment (2.63 D) and enhanced the radical intensity of sodium ascorbate to the greatest extent (unpublished data).

Among the nine different types of cancer cell lines, colon cancer and leukemia were the most sensitive on the basis of GI50 values, and renal, non-small cell lung cancer and ovarian cancer were the less sensitive (Tables IIa and IIb). On the basis of TGI values, colon cancer and melanoma were found to be more sensitive than prostate, ovarian or renal cancer. Based on the LC50 values obtained, the order of the sensitivity of 9 cell lines against the 5 compounds was somewhat similar in TGI values. LC50 values were higher than TGI values in any cases.

Considering these results, we can conclude that an

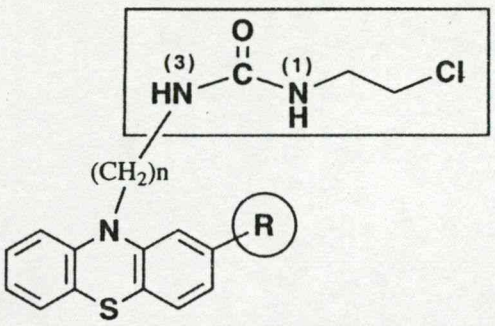

Figure 2. *Structure of phenothiazine skeleton, aliphatic chain and urea site on "half-mustard type" phenothiazines [7-12]. Urea site is indicated by box, and 2-substituted group (R=H, Cl, CF_3) on phenothiazines is circled.*

interaction of the 10th nitrogen (N) of the substituted phenothiazines with DNA, side chain charge and the conformation, and lipophilicity might affect the anticancer effects of the compounds. Moreover, the urea portion of -$N^{(3)}$H-CO-$N^{(1)}$H- of "half-mustard type" phenothiazines synergistically enhanced the lipophilicity of phenothiazine skeleton, making them more permeable through cell membranes (Figure 2). From these theoretical calculations, we have some evidence that the selectivity of the "half-mustard type" phenothiazines might depend on the accessible structure(s) of cell surface membranes of target cells, and on the reactivity to the macromolecules of the cells. Low sensitivity of ovarian cancer cells might possibly be due to the presence of a particular mucin in the cell surface membrane. The possibility that P-glycoproteins might serve as one of such target proteins remains to be investigated.

"Half-mustard type" phenothiazines with buylene linker showed higher antitumor activity than those with propylene linker. It is possible that the anticancer effect of "half-mustard type" phenothiazines might depend on a particular membrane structure in the cancer cells or on the presence of sensitive point in the cell cycle. The present results demonstrated the "cancer type-specific" antiproliferative action of "half-mustard type" phenothiazines [**8-12**]. It is obvious that the colon-cancer, leukemia and melanoma are more sensitive than prostate or ovarian cancer. The compounds might act on the cancer cells *via* alkylurea-induced alkylation of proteins or on DNA by a particular intercalation.

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References

1 Molnár J, Mándi Y, Boda K and Földeák S: The effect of ring-substituted and quaternary chlorpromazine derivatives on the survival of the NK/Ly ascites tumor bearing mice. Neoplasma *29:* 215-222, 1982.

2 (a) Gottlieb LS, Hazel M, Broitman SA and Zamcheck N: Effect of chlorpromazine on a transplantable mouse mastocytoma. Proc Fed Proc *19:* 181, 1960. (b) Gottlieb LS, Broitman SA, Vitale JJ and Zamcheck N: Influence of alcohol and dietary magnesium upon hypercholesterolemia and atherogenesis in the rat. J Lab Clin Med *53:* 433-441, 1959. (c) Gottlieb LS, Broitman SA, Vitale JJ and Zamcheck N: Failure of endogenous serotonin to produce lesions of the carcinoid syndrome. AMA (American Medical Association) Arch Pathol *69:* 77-81, 1960.

3 Motohashi N: Phenothiazines and calmodulin. Anticancer Res *11:* 1125-1164, 1991.

4 Motohashi N, Gollapudi SR, Emrani J and Bhattiprolu KR: Antitumor properties of phenothiazines. Cancer Invest *9:* 305-319, 1991.

5 (a) Cohen MH: Enhancement of the antitumor effect of 1,3-bis(chloroethyl)-1-nitrosourea by chlorpromazine and caffeine. J Natl Cancer Inst *51:* 1323-1325, 1973; (b) Cohen MH: Enhancement of the antitumor effect of 1,3-*bis*(2-chloroethyl)-1-nitrosourea by various psychotropic drugs in combination with caffeine. J Pharmacol Exp Ther *194:* 475-479, 1975. (c) Sobels FH and Mendelson D: Caffeine treatment of the maternal repair system and repair of chromosome breaks induced in Drosophila spermatids. Mutation Res *28:* 133-136, 1975. (d) Mendelson D: The effect of caffeine on repair systems in oocytes of Drosophila melanogaster. Mutation Res *22:* 145-156, 1974. (e) Mendelson D and Sobels FH: The inhibiting effect of caffeine on the maternal repair of radiation-induced chromosome breaks in Drosophila. Mutation Res *26:* 123-128, 1974. (f) Kobayashi T, Yasuda M, Iijima K, Toriizuka K, Cyong J-C and Nagasawa H: Effects of coffee cherry on the immune system in SHN mice. Anticancer Res *16:* 1827-1830, 1996. (g) Hibasami H, Achiwa Y, Fujikawa T and Komiya T: Induction of programmed cell death (apoptosis) in human lymphoid leukemia cells by catechin compounds. Anticancer Res *16:* 1943-1946, 1996.

6 van Woert MH, Palmer SH: Inhibition of the growth of mouse melanoma by chlorpromazine. Cancer Res *29:* 1952-1955, 1969.

7 Aaron P and Levij IS: Antineoplastic effect of chlorpromazine in chemical carcinogenesis in hamster cheek pouch. Cancer Res *32:* 1912-1915, 1972.

8 (a) Motohashi N, Kurihara T, Kawase M, Hevér A, Tanaka M, Szabo D, Nacsa J, Yamanaka W, Kerim A and Molnár J: Drug resistance reversal, anti-mutagenicity and antiretroviral effect of phthalimido- and chloroethyl-phenothiazines. Anticancer Res *17:* 3537-3543, 1997. (b) Motohashi N, Kurihara T, Ghosh S, Dastidar SG, Chakrabarty AN and Molnár J: Relationship between *in vitro - in vivo* antibacterial activity and theoretical calculations of "half-mustard type" phenothiazines. *In:* Chakrabarty AN, Molnár J, Dastidar SG and Motohashi N (eds), Non Antibiotics-A New Class of Unrecognised Antimicrobics. National Institute of Science Communication (NISC), New Delhi, India, 1998, 246-261.

9 Motohashi N, Sakagami H, Kamata K and Yamamoto Y: Cytotoxicity and differentiation-inducing activity of phenothiazine and benzo[*a*]phenothiazine derivatives. Anticancer Res *11:* 1933-1938, 1991.

10 George KC and Singh BB: Synergism of chlorpromazine and

hyperthermia in two mouse solid tumours. Brit J Cancer *45:* 309-313, 1982.

11 Shenoy MA and Singh BB: Cytotoxic and radiosensitizing effects of chlorpromazine hydrochloride in sarcoma 180A. Indian J Exp Biol *18:* 791-795, 1980.

12 Molnár J, Taródi B, Galfi M, Matkovics B and Motohashi N: *In vitro* antiproliferative effects of tricyclic psychopharmacons and synergism with some resistance modifiers. Anticancer Res *12:* 273-280, 1992.

13 Motohashi N: Test for antitumor activities of phenothiazines and benzo[*a*]phenothiazines. (in Japanese) Yakugaku Zasshi *103:* 364-371, 1983.

14 Motohashi N: Antitumor activities of phenothiazines (Phenothiazine Oncology). *In:* Gupta RR ed, Phenothiazines and 1,4-benzothiazines. Chemical and biological aspects. Bioactive Molecules. Elsevier, Vol 4, 1988, pp 705-770.

15 Kidd SE, Nelson MJ, Epstein M, Aszalós A, Pine S and Molnár J: Inhibition of GP 120 and antibodies binding to CD 4 by metal complexes of promazines. Antiinfect Drugs Chemother *15:* 71-77, 1997.

16 Motohashi N, Kawase M, Kurihara T, Hevér A, Nagy S, Tanaka M and Molnár J: Synthesis and antitumor activity of 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas as potential anticancer agents. Anticancer Res *16:* 2525-2532, 1996.

17 Hewlett I, Lee S, Molnár J, Foldeak S, Pine PS, Weaver JL and Aszalos A: Inhibition of HIV infection of H9 cells by chlorpromazine derivatives. J Acquired Immun Deficiecy Syndromes Human Retrovirol *15:* 16-20, 1997.

18 (a) Wuonola MA, Palfreyman MG, Motohashi N, Kawase M, Gabay S, Nacsa J and Molnár J: The primary *in vitro* antitumor screening of "half-mustard type" phenothiazines. Anticancer Res *17:* 3409-3423, 1997. (b) Wuonola MA, Palfreyman MG, Motohashi N, Kawase M, Gabay S, Nacsa J, Gupta RR and Molnár J: The primary *in vitro* anticancer activity of "half-mustard type" phenothiazines in NCI's revised anticancer screening paradigm. Anticancer Res *18:* 337-348, 1998.

19 Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace AJ, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE and Paul KD: An information-intensive approach to the molecular pharmacology of cancer. Science *275:* 343-349, 1997.

20 Wuonola MA, Palfreyman MG, Motohashi N, Kawase M, Gabay S and Molnár J: The *in vitro* antitumor assay of "half-mustard type" phenothiazines in screens of AIDS-related leukemia and lymphomas. Anticancer Res *17:* 3425-3429, 1997.

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Membrane Associated Antitumor Effects of Crocine-, Ginsenoside- and Cannabinoid Derivates

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Abstract. *In the present work a systematic study was initiated with crocine, ginsenoside and cannabinoid derivatives on multidrug resistant mouse lymphoma cells, viral tumor antigen expression and some human leukocyte functions. Among saffron derivatives, crocin and picrocrocin, triglucosyl and diglucosyl crocetin were ineffective on the reversal of multidrug resistance of lymphoma cells. Ginsenoside increased drug accumulation and tumor antigen expression at 2.0-20.0 μg/mL. Some cannabinoid derivatives such as cannabinol, cannabispirol and cannabidiol increased drug accumulation, while cannabidiolic acid, delta-9-THC and tetrahydro-cannabidiolic acid reduced drug accumulation of the human mdr1-gene transfected mouse lymphoma cells. The reversal of multidrug resistance is the result of the inhibition of the efflux pump function in the tumor cells. Crocetin esters were less potent than crocin itself in the inhibition of EBV early antigen expression. However crocin and diglucosyl-crocetin inhibited early tumor antigen expression of adenovirus infected cells, but triglucosylcrocetin was less effective at 0.01-1.0 μg/mL. The crocin had no antiviral effect [on HSV-2 infected vero cells] up to 25 μg/mL concentration. Ginsenosides had a moderate inhibitory effect except ginsenoside Rb1 (was the less effective) on the drug efflux pump. Among the cannabinoid derivatives the cannabinol and cannabispirol increased drug accumulation, while cannabidiolic acid and delta-8-THC, delta-9-THC and tetrahydro- cannabinol reduced drug accumulation in multidrug resistant mouse lymphoma cells. It is interesting that ginsenosides had a chemical structure-dependent immunomodulating effect by enhancing the activity of NK-cells and ADCC activities.*

Various components isolated from medicinal plants have different antitumor effects: *e.g.*, cytotoxic-, chemopreventive-, apoptosis- and differentiation induction. Recently Shoyama and co-workers found that saffron derivatives inhibit chemical carcinogenesis in mice (1).

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Key Words: Multidrug resistance, tumor antigen, immunomodulation, crocine ginzenoside, cannabinoid.

Sangwan *et al* listed the edible plants with potential antimutagenicity and also reported that various kinds of plant products have been studied as antimutagens evidently related to cancer prevention (2).

Among them we selected three traditional medicines, saffron, ginseng and marihuana components for this study. The antitumor activity of saffron in mice transplanted with sarcoma-180, Ehrlich ascites carcinoma and Dalton's lymphoma ascites tumors was studied (3). The inhibitory effects of saffron on chemical carcinogenesis in mice using a two-stage assay system (4) and the effect of crocetin on skin papillomas and Rous sarcoma (5) have also been described. Escribano *et al* (6) reported that crocin inhibits the growth of HeLa cells and suggested apoptosis induction. More recently we found that crocin and crocetin derivatives inhibit skin tumor promotion in mice (1). It is well known that the ginseng extracts prevent the cancer disease in Chinese traditional medicine. Konoshima and his co-workers investigated the inhibitory effects of a ginseng water extract and ginsenoside Rg1 for anti- tumor-promoting activities using an *in vitro* two-stage carcinogenesis assay system (7).

The effects of marihuana components was described on the plasmid mediated bacterial resistance and pathogenicity in model experiments (8) and on *E. coli* adhesion to tissue culture cells (9).

Based on the similarity of drug resistance mechanisms in bacteria and cancer cells, it was worthwhile to study the activity of some cannabinoids, ginsenoside and saffron derivatives on the mdr-glycoprotein of cancer cells and tumor antigen expression of virus infected cells. Here we report the effects of crocin, cannabinoids, ginsenoside derivatives.

Materials and Methods

Chemicals. Extraction and separation of active anti-tumor compounds: Dried saffron (500 g) was extracted with 50 % EtOH. The EtOH extract (ECS) (311 g) was separated by Silica-gel column chromatography using an EtOAc-EtOH-H20 gradient solution (9:3:1 to 7:3:2) to separate the carotenoid containing fraction (275 g) and non-carotenoid fraction (36 g). The carotenoid containing fraction was repeatedly purified by Silica-gel column chromatography, with an EtOHAc-EtOH-H_2O gradient solution (9:3:1 to 7:3:2), and finally purified by a Mel gel column

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chromatography elution with an H_2O-MeOH gradient solution (1:0 to 0:1) to yield crocetin di-glucose ester (0.41 g), crocetin gentibiose glucose ester (5.10 g) and crocin (5.69 g).

Ginsenosides, Rb1, Rc, Rd, Re and Rg1 were isolated from white ginseng by repeated column chromatography on Silica-gel as previously reported (10). They were directly identified with authentic samples.

Cannabis: delta8-tetrahydrocannabinol (Δ8-THC), delta9-tetrahydrocannabinol (Δ9- THC), cannabinol and cannabidiol were kindly supplied by Dr. Keizo Watanabe, narcotics Laboratory Section, Division of Narcotic Drugs, United Nations, Vienna Intemational Center, Vienna, Austria. Tetrahydro-cannabidiolic acid (THCA) triethylamine was prepared by Alkaloida, Tiszavasvari, Hungary.

The compounds were dissolved in DMSO; final concentrations were 1.0, 5.0 and 10 μg/mL, in all triplicate test cultures. Both control cultures and test cultures contained DMSO (Sigma) in the same concentration.

Cell cultures. For the cytotoxicity assay, Raji cells and Hep-2 cells were cultivated in RPMI supplemented with 10 % foetal calf serum. The L5178Y (parent) mouse T-cell lymphoma and its transformed subline with mdr (MDR1/A) were grown in McCoy's 5A medium supplemented with 10% heat-inactived horse serum. HEp-2 cells for immunofluorescence studies were grown on glass coverslip in Petri dishes in Eagle MEM supplemented with 5 % new born calf serum.

For T-antigen expression the highly oncogenic, human adenovirus of serotype 12 was applied. Infectivity of the virus suspension was determined and found to be $10^{5.5}$ $TCID_{50}$ /mL (12).

Virus and cell culture for antiviral effects. Vero cells were cultured and maintained in RPMI 1640 medium supplemented with 5 % or 2 % calf serum respectively. The virus used was a strain of HSV-2 and it was propagated in Vero cells and infectivity was measured in the same cells by the dilution method on microtitre plates (Linbro, Greiner). The infective titre was expressed as $TCID_{50}$ (50 % tissue culture infective dose) calculated by the formula of Reed and Muench.

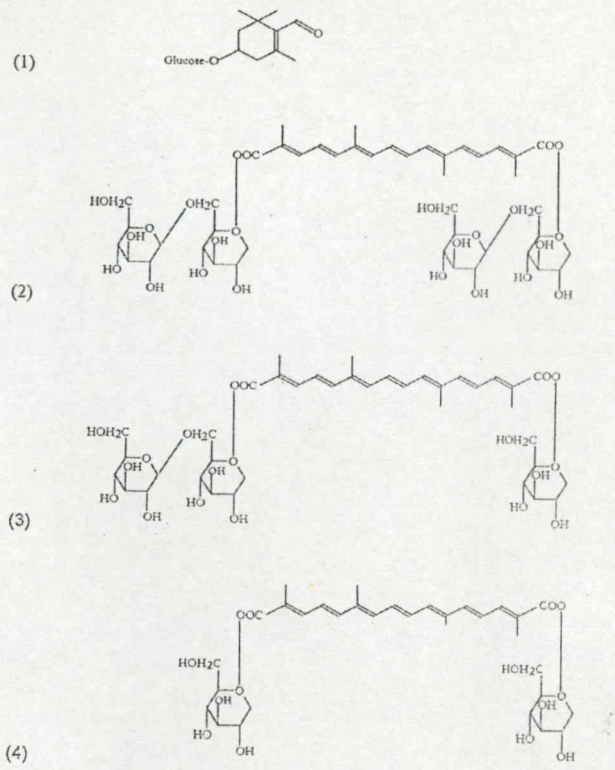


Figure 1. *Chemical structures of picrocrocin (1), crocin (2), crocetin gentiobiose glucose ester (3) and crocetin di-glucose ester (4).*

Reversal of multidrug resistance. The L5178 mouse T cell lymhoma cell line was infected with the pHa MDR1/A retrovirus as previously described (14). MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line, and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cell suspensions were adjusted to a density of $2x10^6$/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquots to Eppendorf centrifuge tubes. Then the tested compounds were added (0.2-100.0 μL of the 1.0 mg/mL stock solutions) and the samples were incubated for 10 min at room temperature. Then 10 μL (5.2 μM final concentration) of the indicator Rhodamine 123 was added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the Rhodamine 123 exclusion experiments. The percentage of control mean fluorescence intensity was calculated for parental and mdr cell lines as compared to untreated cells. An activity ratio was calculated by the following equation (13) on the basis of measured fluorescence values:

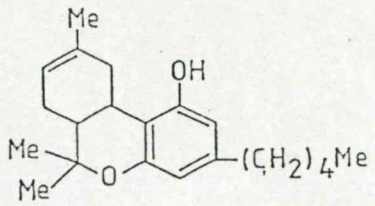
$$R = \frac{\text{mdr treated / mdr control}}{\text{parental treated / parental control}}$$

EVB-EA activation in vitro. The inhibition of EBV-EA activation was assayed using the same method described previously (14). The Raji cells were incubated at 37°C for 48 h in a medium supplemented with n-butyric acid (4 mol), TPA (32 pmol) and various amounts of the test compounds. Smears were made of the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunfluorescence technique. In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was compared with that of the control experiment (100%) with n-butyric acid plus TPA. In the experiments, the EBV-EA, activities were originaly around 40 %, and these values were taken as the positive control (100 %). The viability of cells was assayed against treated cells by the tripan-blue staining method.

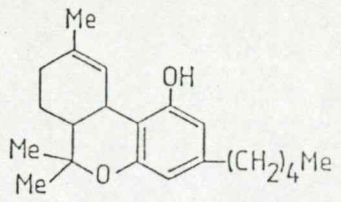
Assay for adenovirus early T-antigen expression by immunofluorescence. HEp-2 cells on the coverslip were infected with adenovirus at a multiplicity of 7 x 10^{-2} and incubated at 37°C for 4 h. After 1 h adsorption, the unadsorbed virus was removed and the cells were washed 3 times with cold medium to remove the unattached virus. The cells were then overlaid with a warm medium containing the appropriate concentration of crocin derivatives. After 16 h incubation, the cells were washed twice with the cold phosphate buffered saline (PBS) and fixed with cold acetone for 20 min at -20°C. The fixed cells were stored at -20°C until the immunofluorescence assays were performed.

T-antigen-producing cells were detected by means of indirect immunofluorescence. Cells were incubated with hamster anti-T serum (diluted 1:15 in PBS) containing 1 % foetal calf serum for 60 min at 37°C. They were subsequently washed with cold PBS and incubated for 45 min at 37°C with fluorescein conjugated rabbit anti-hamster immunoglobulin (diluted 1:30 in PBS). T-antigen-positive cells were scored under LEITZ UV microscope, counting a total of 600 individual cells (12).

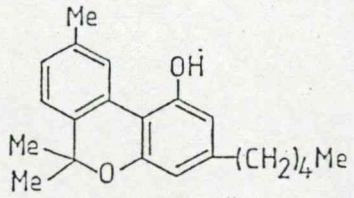
Assay of antiviral activity. Antiviral activity of compounds was investigated by the yield reduction method. Monolayer cultures of Vero



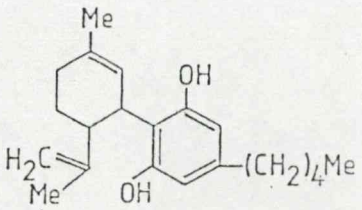
Δ 8 - tetrahydrocannabinol



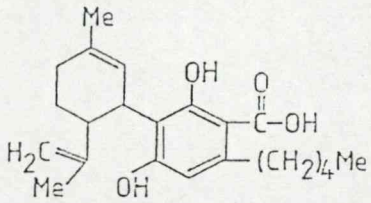
Δ 9 - tetrahydrocannabinol



Cannabinol



Cannabidiol



Cannabidiolic acid

Figure 2. *Cannabinoid derivatives.*

cells were grown in 96-well microtitre plates and were infected with HSV-2 at a multiplicity of 0.5 $TCID_{50}$ per cell. After adsorption for 1 h at 37°C the inoculum was removed and the cultures were washed twice with PBS solution then supplied with RPMI 1640 medium containing 2 % calf serum and the drugs in different concentrations. Virus infected control cells were cultured in the medium without drugs. After incubation for 24 h at 37°C, the cultures were frozen and thawed. The cell debris was removed by low-speed centrifugation. The virus yield in the supernatant fluids was determined in Vero cells grown on microtitre plates by the dilution method. The inhibitory effect of drugs on the virus multiplication was evaluated by comparing the virus titres obtained in the presence and absence of the compounds.

Assay for cytotoxicity. The HEp-2 cells, L5 1 78Y parent and multidrug-resistant (mdr) cells were seeded into Greiner 96-well flat bottom microtrays at 5000 cells/0.1 mL of McCoy's medium supplemented with 10 % horse serum (for L5178) or RPMI medium (for HEp-2) containing 10 % foetal calf serum. The cells were allowed to attach and spread on the bottom of the wells for 6 h. Then 0.1 mL of diluted test compounds was added to the wells. Control cells received 0.1 mL of growth medium, and medium control received 0.2 mL of growth medium without cells. The total volume of medium / well was 0.2 mL. The trays were incubated further at 37°C for 3 days in a CO_2 incubator. Then, the culture medium was discarded and the cells were fixed with 0.05 ml of 10 % formalin for 10 min, followed by staining with 0.05 mL of 0.05 % crystal violet for 30 min. The stained cells were rinsed with tap water and air dried. The dye was dissolved in 0.15 mL of 50 % ethanol plus 0.1 % acetic acid and measured in Elisa-reader.

Antibody dependent cellular cytotoxicity (ADCC) test. Human 0 Rh-positive red blood cells were used as a target and PBM Mo^- cells as an effector in a 1:10 ratio. The reaction was mediated by red blood cell specific anti-D antibodies. The cultures were incubated at 37°C for 16 h and the amount of released ^{51}Cr in each supernatant was determined using a gamma counter. From the average of triplicate released values, the percentage of cytotoxicity was calculated according to the following formula (15).

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}$$

Spontaneous release indicated cultures without anti-D antibody. The results are expressed in percent of control cultures.

Natural Killer cell activity (NK assay). ^{51}Cr-labelled K562 cells (used as the target), and PBM-Mo^+ and PBM-Mo^- (used as the effector) were mixed in a ratio of 1:50. After incubation at 37°C for 4 h, the amount of radioactivity released into culture supernatants was determined. The results are expressed as a percentage of cytotoxicity, as described above (16).

Table I. *Percentage of EBV-EA induction in the presence of crocetin derivatives from Crocus sativus.*

Compounds	Concentration (mol ratio / TPA)[a] EBV-EA	
	1×10^3	1×10^2
Crocin	19.5[b] (>80)[c]	82.7 (>80)
Triglucosyl crocetin	30.1 (>80)	100.0 (>80)
Diglucosyl crocetin	37.6 (>80)	100.0 (>80)

[a] TPA (20 ng = 32 pmol).
[b] Values represent relative percentage to the positive control value (100 %).
[c] Values in the parentheses are viability percentage of Raji cells.

Table II. *The effect of crocin and its derivatives on the expression of early tumor antigen of adenovirus 12 infected Hep-2 cells.*

Compounds	Concentration µg/ml	Expression of adeno EA in percent of the control
Crocin	0.01	100
	0.10	5 - 10
Diglucosyl-crocetin	0.01	10
	0.10	5
Triglucosyl-crocetin	0.01	100
	0.10	100
	1.00	5 - 10
Control	-	100

Results

The primary screening of crocin was carried out with a short-term *in vitro* assay on EBV-EA activation. The inhibitory effects of crocetin glycosides on activation induced by TPA and viability of Raji cells are shown in Table I.

Table I shows the inhibitory effect of compounds isolated from active carotenoid fraction against EBV-EA induction. In the ingredients of the ethanol extract of *Crocus sativus* (ECS) crocetin gentibiose glucose ester and crocetin di-glucosyl ester, were less potent than crocin in inhibiting the EBV-EA induction effect. The comparative study among three analogues clearly suggests that gentibiose attached to the diterpenoid chain are important for crocin to exert the biological activity.

The exposure of the EBV genome carrying lymphoblastoid Raji cells derived from Burkitt lymphoma and exposure of adenovirus infected cells by some compounds resulted in altered T-antigen expression in HEp-2 cells (Table II). The crocin and diglucosyl-crocetin inhibited early tumor antigen expression in HEp-2 cells in the presence of triglucosyl-crocetin the effect was less significant. Interestingly, the crocin had no antiviral effect on herpes simplex virus-type-2 (Table III). These findings led us to suppose that crocetin glucoside may affect the activity of membrane proteins such as mdr p-glycoprotein (Table IV).

The mdr glycoprotein located in the membrane of tumor cells are responsible for the decreased retention of a wide variety of anticancer drugs in cells. Therefore, the phenomenon is called multidrug resistance, which means cross-resistance for a large number of antitumor drugs.

Therefore, the main aim of our studies in this paper was to, improve the strategies designed to block the activity of ppg membrane protein to circumvent a form of drug- resistance by membrane active compounds like ginsenosid and cannabinoid derivatives.

The resistance reversal effect of crocetin glycosides was tested on mouse lymphoma cells carrying the human mdr1 gene. The data obtained with crocetin glycosides is summarized in Table IV. The triglucosyl-crocetin and diglucosyl-crocetin activity was marginal for 0.1 to 10.0 µg/mL concentration; however, picrocrocin and crocin were ineffective in this aspect. Even drug accumulation was decreased in the treated cells. Thus, one cannot exclude the enhanced membrane permeation or apoptosis.

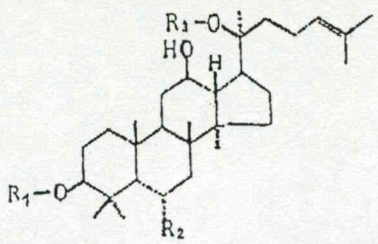
Table III. *The antiviral effect of crocin on HSV-2.*

Compounds	Concentration µg/ml	Inhibition (log)
Crocin	50	0.49
	25	0.24
	12.5	0.0
	6.25	0.0
	3.12	0.0
	1.56	0.0

The interpretation of multidrug resistance reversal effect of the compounds also gives some information about direct cytotoxic potential. The morphological changes were analysed by measuring the FSC and SSC values on the flow cytometer.

The cell size increased, and at the same time cellular granulation decreased. The viability of cells did not change during the experiment. As determined by the trypan blue test, the cell viability was between 95 and 98 % (data not shown).

Some ginsenoside derivatives had a remarkable inhibition on mdr efflux, and the effect also showed a dose dependence



Ginsenoside	R_1	R_2	R_3
Rb_1	Glc---Glc-	H	Glc----Glc--
Rc	Glc---Glc-	H	Ara(f)---Glc-
Rd	Glc---Glc-	H	Glc-
Re	H	Rha---Glc-O-	Glc-
Rg_1	Glc---Glc-	Glc-O-	Glc-

Figure 3. *Ginsenoside derivatives.*

Table IV. *The reversal of multidrug resistance of mouse lymphoma cells by crocetin glycosides and picrocrocin.*

Compounds	Concentration µg/ml	FSC	SSC	FL-1	Fluorescence Activity Ratio
PAR control		548.98	170.71	743.22	
MDR control		649.28	259.87	36.03	
Verapamil	8	651.43	255.02	185.33	5.14
Crocin	10	695.35	235.23	23.25	0.65
	20	684.18	230.62	17.14	0.48
	100	687.09	231.37	13.54	0.38
Triglucosyl crocetin	10	708.07	220.63	40.79	1.13
	20	678.15	219.71	43.18	1.20
	100	682.96	219.59	37.77	1.05
Diglucosyl crocetin	10	697.47	230.71	22.45	0.62
	20	698.58	225.24	44.74	1.24
	100	695.02	239.36	32.68	0.91
Picrocrocin	10	693.62	224.73	27.28'	0.76
	20	689.09	225.03	25.78	0.2
	100	665.07	219.06	25.12	0.70

Table V. *The reversal of multidrug resistance of mouse lymphoma cells by naturally occurring glycosides.*

Compounds	Concentration µg/ml	FSC	SSC	FL-1	Fluorescence Activity Ratio
PAR control		522.20	260.98	1010.84	
MDR control		581.72	311.94	47.57	
Verapamil	8	578.25	312.83	374.40	7.87
Ginsenoside Rg1	2	589.12	315.78	99.32	2.09
	20	585.34	307.60	113.27	2.38
Ginsenoside Rb1	2	585.29	304.65	75.78	1.59
	20	585.02	298.95	87.95	1.85
Ginsenoside Re	2	586.11	300.44	142.80	3.00
	20	588.32	301.59	145.84	3.07
Ginsenoside Rc	2	589.62	308.93	245.17	5.15
	20	584.74	299.28	182.13	3.83
Ginsenoside Rd	2	582.43	300.19	278.08	5.85
	20	585.17	307.34	363.94	7.65

Table VI. *The reversal of multidrug resistance of mouse lymphoma cells by some cannabis derivatives.*

Compounds	Concentration µg/ml	FSC	SSC	FL-1	Fluorescence Activity Ratio
Verapamil	5	528.86	253.87	62.89	3.15
Cannabinol	2	502.23	262.80	221.29	11.08
Cannabidiol	2	502.93	235.87	57.79	2.89
Cannabidiolic acid	2	490.11	248.36	10.91	0.55
Δ 8 THC	0.5	497.26	246.43	13.58	0.68
Δ 9 THC	0.5	491.21	244.91	14.45	0.72

for 2.0 to 20.0 µg/mL. The ginsenoside Rc and Rd had the highest effect by reducing the activity of drug efflux pump. These two ginsenosides had nearly the same effect without any apparent cytotoxicity (Table V).

Some of the cannabinoid compounds showed a remarkable inhibition on the Rhodamine accumulation of tumor cells at 0.5-5.0 µg/mL concentrations (Table VI). We could not detect any inhibitory effect in case of the hallucinogenic Δ8-Δ9 THC.

Table VII. *Effect of the drugs on NK cells activity.*

Compounds	NK cell activity (% of control value)	
	1 μg/ml	10 μg/ml
Ginsenoside Rgl	91	117*
Ginsenoside Rbl	106*	114*
Ginsenoside Re	93	85
Ginsenoside Rc	85	103*
Ginsenoside Rd	97	116*
Control values DMSO	17.9	15.2

Table VIII. *ADCC activity in the presence of the drugs.*

Compounds	ADCC activity (% of control)		
	1 μg/ml	5 μg/ml	10 μg/ml
Ginsenoside Rgl	53	63	95
Ginsenoside Rbl	63	71	88
Ginsenoside Re	55	51	46
Ginsenoside Rc	53	84	97
Ginsenoside Rd	48	63	76
Control values DMSO	66.2	52.4	43.9

The Δ8-THC had a marginal effect at cytotoxic concentration while Δ9 had a remarkable effect at the same, 2.0 μg/ml, concentration, which lead to 10 percent reduction of the cell size without any apparent changes in SSC values or cell viability. It is interesting that the two effective compounds enhanced the NK cell activity (Table VII). That means that ginsenoside Rd and Rg1 may have a beneficial effect in tumor bearing animals. However, the ADCC activity of leukocytes was hardly-enhanced by ginsenoside Rc, Rg1 and Rb1 (Table VIII).

Discussion

Crocus sativus grows in South Asia and South Europe. Its medical value was recorded in traditional Chinese medicine and was used to improve blood circulation (17). The compounds of saffron are able to inhibit chemical carcinogenesis as chemopreventive agents and had direct antitumor action in animal experiments (3, 18). *Crocus sativus* extracts were effective in the treatment of central nervous system diseases including depression, anxiety, and improved memory functions (19).

It was shown, that the saffron extracts contain characteristic compounds such as carotenoids; crocin and monoterpene aldehydes; picrocrocin and safranal (20). The majority of crocetin glycosides were able to inhibit the growth of human cancer cells *in vitro* (6).

In addition, some plant phenolics were able to enhance the survival time of mice inoculated with NK/LY ascites tumor cells (21, 22); others affected the genetic base of bacterial resistance, including plasmid transfer, which was inhibited by psychoactive and inactive derivates as well (8).

Crocine had an inhibitory effect on two stage carcinogenesis with DMBA as an initiator and TPA as a promoter. When crocin was applied before the TPA treatment only 10 % of animals had papillomas and the formation of papillomas was delayed (1). Konoshima assumed that there are two possibilities by which crocin inhibits tumor formation: a., prevention of DNA damage and b., working as an activator for DNA excision repair enzyme.

There were no data about the effects of these plant compounds on drug resistant cancer, although the molecular mechanism of the membrane effect was analysed. Abe *et al* (23) suggested that crocin antagonizes the ethanol effect on N-methyl-D-aspartate receptor activated membrane current (23). It was interesting that EBV and adenovirus induced tumor- antigen expression was reduced by crocin derivatives, despite the lack of the antiviral effect on the DNA containing herpesvirus.

It is possible that gene expression is blocked due to the reduced promoter activity in the presence of crocin, or that a T-antigen shift can be the consequence of physicochemical changes in the membrane lattice induced by a charge transfer complex formation between particular membrane components and crocin derivatives.

The effect of ginsenoside derivatives was dependent on their chemical structure. The compounds in general had a moderate effect on the inhibition of multidrug resistance efflux pump of the human mdr-1 transfected mouse lymphoma cells. The ginsenoside Rb1 was an exception, having the lowest effect possibly due to the bulky substitution at the C-3 site. This hypothesis was supported by the highest activity of Rd derivative. In the latter compound the space-filling of the C-3 substituent is much smaller than in Rb1. However, the moderate action of the Rg1 may be due to steric hindrance problems at the C-6 position.

In the case of cannabinol, the electrophilic nature of the molecule may be responsible for the reversal of the multidrug resistance efflux-pump. However, the extreme hydrophobicity and broken pi-conjugation in the molecules of the two hallucinogenic compounds such as Δ8 and Δ9 THC might be responsible for their ineffectiveness. The tested compounds can change the membrane structure and only a few of them are able to block the mdr-efflux pump.

In addition, cannabinol can enhance the phagocytic index

and TPA-induced chemiluminescent reaction of human polymorphonuclear leucocytes by enhancing oxidative bursts (9). The relevant immuno-modulating effect of the most active ginsenoside was studied in two functions of the human leucocytes such as NK cell activity and ADCC. The ginsenosides were able to enhance the natural killer cell activity, however the ADCC activity changed to a lesser extent.

In conclusion, we suggest that the reduced T-antigen expression, increased natural killer cell activity and inhibition membrane efflux pump responsible for the multidrug resistance can be exploited in antitumor chemotherapy.

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References

1 Konoshima T, Takasaki M, Tokuda H, Morimoto S, Tanaka H, Kawata E, Xuan LJ, Saito H, Sugiura M, Molnár J, Shoyama Y: Crocin and crocetin derivatives inhibit skin tumor promotion in mice. Phytotherapy Res *12:* 400-404, 1998.

2 Sangwan NS, Shanker NS, Sangwan RS, Kumar S: Plant-derived products as antimutagens. Phytotherapy Research *12:* 389-399, 1998.

3 Nair SC, Pannikar B, Panikkar KR: Antitumor activity of saffron (*Crocus sativus*). Cancer Lett *57:* 109-114, 1991.

4 Salomi MJ , Nair SC , Panikkar KR: Inhibitory effects of *Nigella sativa* and saffron on chemical carcinogenesis in mice. Nutr Cancer *16:* 67-72 1991.

5 Gainer JL , Wallis DA , Jones JR: The effect of crocetin on skin papillomas and rous sarcoma. Oncology *33:* 222-224, 1976.

6 Escribano J, Alonso GL , Coca-Prados M, Fernandes JA: Crocin, safranal and picrocrocin from saffron (*Crocus sativus L.*) inhibit the growth of human cancer cells *in vitro*. Cancer Lett *100:* 23-30, 1996.

7 Konosima T, Takasaki M, Tokuda H: Anti-tumor-promoting activities of the roots of *Panax notoginseng.* Natural Med *50:* 158-162, 1996.

8 Molnár J, Nakamura MJ : Effect of cannabis derivatives on plasmid replication in *Escherichia coli.* Res Commun Substances of Abuse *6:* 23-35, 1985.

9 Molnár J, IB Petri, I Berek, Y Shoyama, I Nishioka: The effect of cannabinoids and cannabispiro compounds on E. coli adhesion to tissue culture cells and on leukocyte functions *in vitro*. Acta Microbiol Acad Sci Hung *34:* 233-240, 1987.

10 Kitagawa I, Yoshikawa M, Yoshihara M, Hayashi T, Taniyama T: Chemical studies of crude drugs (1). Constituents of *Ginseng radix rubra*. Yakugaku Zasshi *103:* 612-622, 1983.

11 Molnár J, Pusztai R, Hever A, Nagy Sz , Motohashi N: Effect of two benzo[a]phenothiazines on multidrug resistance and tumor antigen expression. Anticancer Research *15:* 2013-2016, 1995.

12 Pusztai R, Motohashi N, Párkányi C, J-J Aaron B., Kesava Rao, Molnár J,: Relationship between tumor antigen expression and substituent effect on benzo[a]phenothiazines. Anticancer Res *16:* 2961-2964, 1996.

13 Cornwell MM, Pastan I, Gottesman MM: Certain calcium channel blockers bind specifically to multidrugresistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem *262:* 2166-2170, 1987.

14 Konoshima T, Koyama KM, Okatani T, Tagahara K, Tokuda H: Studies on inhibitors of skin tumor promotion. VI. J. Natl Prod *52:* 987-955, 1989.

15 Petri IB , Lórincz A, Berek I: Further investigation of nonspecific substances in anti- Rh(D) preparation. Vox Sang *51:* 287-291, 1986.

16 B Petri, E Szekeres, I Berek, Molnár J, H Sakagami, N Motohashi: Effect of benzo[a]phenothiazines on Natural Killer cell activity of peripheral blood mononuclear cells. Anticancer Res *13:* 2273-2276, 1993.

17 Gainer J, Jones JR: The use of crocetin in experimental atherosclerosis. Experientia *31:* 548-549, 1975.

18 Salomi MJ , Nair SC , Panikkar KR: Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. Nut Cancer *16:* 67-72, 1991.

19 "Dictionary of Traditional Chinese Medicine (Zhong Yao Da Zi Dian)", Vol. 2, ed. by Suzhou New Medical College, Sanghai People's Publication House, Shanghai, 2622-2623, 1977.

20 Alonso GL , Varon P, Navarro FF, Salimas MR: Autooxidation in saffron at 40°C and 75 % relative humidity. Food Sci *55:* 595-596, 1990.

21 Molnár J, Beladi I, Domonkos K, Földeak S, Boda K, Veckenstedt A: Antitumor activity of flavonoids on NK/LY ascites tumor cells. Neoplasma *28:* 11-18, 1981.

22 Molnár J, Mucsi I, Mandi Y, Beladi I, Foldeak S, Boda K: The effect of rutin-N- mustard on the survival of NK/LY ascites tumor-bearing mice. Int J Cancer *30:* 767-771, 1982.

23 Abe K, Sugiura M, Shoyama Y, Saito H: Crocin antagonizes ethanol inhibition of NMDA receptor-mediated responses in rat hippocampal neurons. Brain Res *787:* 132-138, 1998.

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Review

Reversal of Multidrug Resistance of Tumor Cells

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Abstract. *Drug resistance to chemotherapy is rapidly emerging. Resistance to one drug carries over resistance to unrelated anticancer drugs leading to multidrug resistance (MDR). A major factor of MDR is P-glycoprotein (P-gp) mediated ABC transport found in many eukaryotic cells. P-gp acts as a drug eMux pump. The mdr1 gene involved in P-gp 170 protein production is localized in the human chromosome 7 band p2 1.0 - 21.1. Point mutations alter cross-resistance patterns. A variety of stimuli increase the expression of the mdr1 gene: lowered extracellular pH, heat shock, arsenite, cytotoxic agents, anticancer drugs, transfection with oncogenes, HIV-I, and UV-irradiation. An alternative hypothesis to the efflux pump claims that P-gp modifies the intracellular environment to reduce accumulation of anticancer drugs in cancer cells by creating ionic or proton gradients. Chemosensitizers that block P-gp drug extrusion are generally lipid-soluble at physiological pH, possess a basic nitrogen atom and at least two co-planar rings. P-gp blocking does not depend on drug chirality. This opens the way of treating P-gp related MDR with chiral versions of drugs relatively harmless in terms of side-effects. We believe that resistance modifiers combined with cytostatics will chemotherapeutically be more effective for cancer patients.*

Cancer is the second most frequent disease globally. The hope for cures of most cancers are probably systemic treatments such as chemotherapy and immunotherapy. Chemotherapy has proven to be effective and has led to the cure of many cancers such as leukemias, lymphomas and sarcomas. The common clinical problem in the successful treatment of cancer is the resistance of tumor cells to chemotherapeutic drugs.

Resistance to chemotherapeutics has recently been observed to be emerging among viruses, bacteria, fungi, protozoa and cancer cells. The basic reason for the development of this resistance is a Darwinian selection, which ensures the accommodation of living organisms to an altered environment (1). During the last 20 years drug resistance mediated by a membrane glycoprotein has been studied in detail. This type of resistance has a special importance because cancer cells exposed to one cytostatic results not only in a specific resistance to the inducer, but also to many other chemically unrelated anticancer drugs representing a non-specific cross-resistance.

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The ABC superfamily of membrane transporter proteins

P-glycoprotein belongs to a large group of functional proteins that share common structural and functional properties. This superfamily of proteins has been named the ABC (ATP Binding Cassette) family of membrane traffic ATPases. There are at least 40 members of this ABC superfamily of proteins in bacteria, including nutrient, peptide, polysaccharide, toxin and drug transporters. There are many examples of these ABC proteins in eukaryotic cells: a pigment transporter in *Drosophila melanogaster* (2), a pump that appears to mediate chloroquine resistance in *Plasmodium falciparum*, pfmdr (3,4), a transporter for a peptide mating factor of yeast called STE6 (5,6); CFTR, the product of the cystic fibrosis gene (7,8), a peroxisomal membrane pump in the liver (9) which, if it is mutated results in a fatal cerebro-hepato-renal dysfunction (10); and two linked genes associated with the transport of peptides into the endoplasmic reticulum for class I antigen presentation, recently named Tap-1 and Tap-2 (11).

The general structure of these transporters includes a set of six transmembrane domains, which are generally not homologous to others sharing amino acids with hydrophobic properties, followed by an ATP binding casette. In the bacterial systems, the subunit with the transmembrane domains and the ABC proteins may be separate or fused. In eukaryotic systems, a set of six transmembrane domains may be fused to a single ABC protein, as for the peroxisomal membrane protein, endoplasmic reticulum peptide transporters, *Drosophila* pigment transporter and the antigen

Table I. *Mammalian multidrug resistance genes.*

Species	Class I	Class II	Class III
Human	MDR1		MDR3 (MDR2)
Mouse	mdr3 (mdr1 a)	mdr1 (mdr1 b)	mdr2
Hamster	Pgp1	Pgp2	PgP3
Rat	Pgp1	Pgp2 (mdr1 b)	pgp3 (mdr2)

peptide transporters, or two of these may be fused to give 12 transmembrane domains and two ATP sites, such as the CFTR, STE6 and MDR proteins. Although this family has not yet been fully described, it appears that the ABC transporters localised to intracellular membranes contain only one set of six transmembrane domains and one ATP site, whereas the transporters localised to the plasma membrane have 12 transmembrane domains and two ATP sites. Several secondary transporters such as the glucose carrier, anion exchanger and Na/H exchanger, which do not have ATP sites, when localised to the plasma membrane also contain 12 transmembrane domains, but homologous intracellular organelle transporters contains six transmembrane domains and function as dimers (12,13). Based on this analogy, it is tempting to suggest that the minimal functional unit of the ABC transporters may also require 12 transmembrane domains and two ATP sites.

Broad-spectrum resistance to chemotherapy in human cancers has been called multidrug resistance (MDR). This resistance is due to decreased accumulation of drugs in cells, and the efflux of cytostatics is mediated by an energy-dependent drug transporter protein (2). One of the biochemical aspects by which tumor cells manifest multidrug resistance is the overexpression of an integral plasma membrane P-glycoprotein or P-170, the product of an SOS gene. This is the multidrug resistance 1 (MDR1) gene. P-glycoprotein (P-gp) acts as a drug efflux pump that actively extrudes drugs from tumor cells, thereby decreasing the concentration of chemotherapeutic agents in resistant cancer cells or HIV-1 protease inhibitors (14).

The overexpression of the MDR1, MRP and LRP genes explains only a subset of multidrug resistance. More studies have identified resistance-related abnormalities of the enzyme topoisomerase II (15,16) with respect to overexpression of the genes involved in the glutathione S-transferase system (17,18) and the expression of other genes (19,20). These studies have provided additional mechanisms by which multidrug resistance can be conferred. Even though these phenomena are associated with multidrug resistance, the breadth of the resistance associated with each is not as broad as the MDR now often encountered in the clinic.

Table II. *Potential physiological functions of P-gps in mammals (from Borst and Schinkel, 1996).*

1. Protection against exogenous toxins ingested with food:
   Expression in small intestine, colon, blood-tissue barrier sites.
2. Excretion of metabolites or toxins:
   Expression in liver canalicular membrane, kidney.
3. Transport of steroid hormones:
   Expression in adrenal gland, demonstrated transport of cortisol, corticosterone, aldosterone.
4. Extrusion of (poly-)peptides (cytokines) not exported from the cell *via* the classical signal/cleavage pathway:
   Compare yeast St6, E. coli HlyB, mammalian endoplasmic reticulum peptide transporters.
5. Ion transport and cell volume regulation:
   Activation of an endogenous Cl^- channel activity.
6. Lymphocyte cytotoxicity:
   Possible involvement in NK-cell-mediated cytotoxicity.
7. Transport of prenylcysteine methyl esters.
8. Intracellular vesicular transport of cholesterol.

Genes responsible for MDR

Different P-gp isoforms have been identified, and these are encoded by a family of closely related genes. They are referred to as pgp genes in hamsters and rats, mdr genes in mice and MDR genes in man (21). Based on their 3'-untranslated regions the mammalian multidrug resistance genes are divided into three classes of genes, termed class I, class II and class III as in Table I (22).

The rodent class I and class II genes appear to be more closely related to each other in structure and function than either gene to the class III gene. Overexpression of class I or class II P-gps renders cells multidrug resistant, while class III P-gps is not capable of conveying multidrug resistance. The structural similarities between two halves of the transmembrane P-gp molecule led to the hypothesis that the P-gp gene family arose from the duplication of a primordial gene (23). However, detailed analysis of the MDR1 exon/intron structure indicated that this is probably not the case (24).

The MDR1 gene has been localized in the human chromosome 7 band p21-21.1 (25) on a 600-kb NruI fragment and the entire MDR1 coding region is contained on a 120-kb XhoI fragment (26). This gene extends over more than 100 kb and encompasses 28 introns, 26 of which interrupt the protein-coding sequence. Its messenger RNA has a size of 4.7kD so its coding region therefore represents less than 5 %

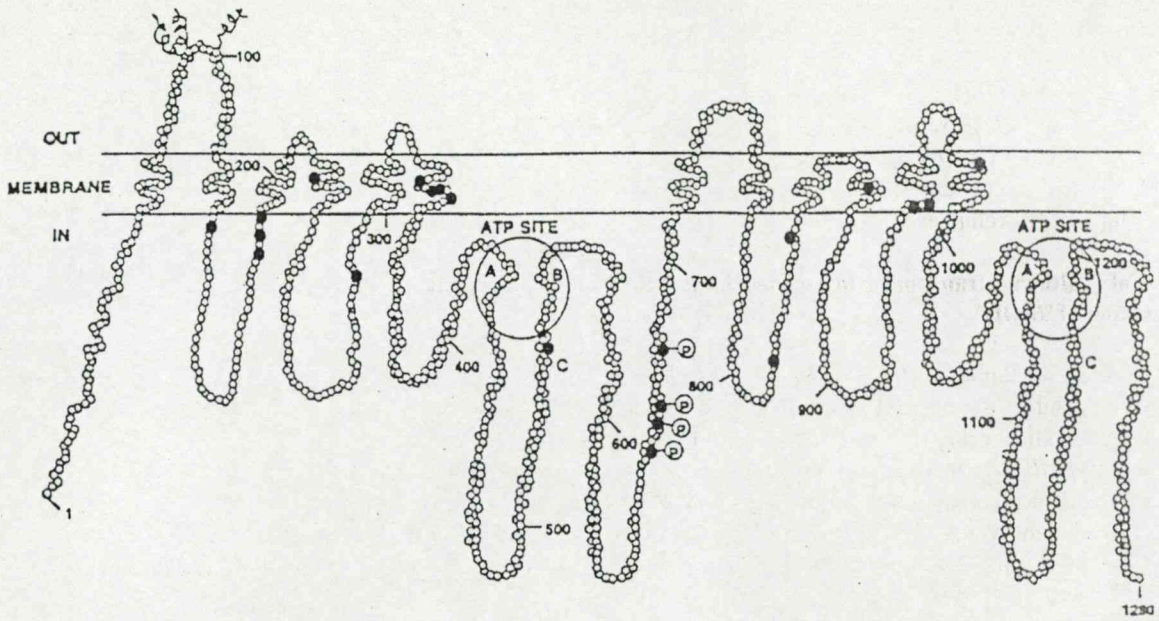


Figure 1. *Schematic model of the structural organization of human multidrug transporter, based on amino acid sequence.*

of its total length. The human MDR2 gene has the same number of 28 exons, 27 coding, but is shorter and only extends for 74 kb. The function of the closely related MDR2 genes is not known and its substrates have not yet been identified (27).

One of the most intriguing aspects of P-gp biology is the broad substrate specificity of the molecule, which can be altered by point mutations. Spontaneous base pair substitutions in the human MDR1 gene, resulting in a glycine to valine change at amino acid 185, located at the cytoplasmic border of the third transmembrane domain, change the pattern of resistance by their increased ability to pump colchicine and etoposide, with a decrease in the ability to pump vinblastine and actinomycin D (28). Both intact halves of the P-gp molecule, as well as the two nucleotide binding sites, seem to be essential for drug transport (29). Evidence is accumulating that transmembrane domains of P-gp are important for the interaction of chemotherapeutic drugs with P-gp (30). For example, predicted transmembrane domain 3, 6 and 11 are likely to play a role in substrate recognition, binding or release. Point mutations, either spontaneously occurring or genetically engineered, within or in close proximity to these putative transmembrane domains, alter cross-resistance patterns. An important role for the transmembrane domains of the P-gp molecule for drug transport fits with the hydrophobic vacuum cleaner model of the function of P-gp (31). As a consequence of its broad substrate specificity, the function of the P-gp molecule can be inhibited by non-cytotoxic compounds that also have a high affinity for the drug binding site on the P-gp.

Sequence, structure, expression andfunction of P-glycoprotein

P-gp is 1280 amino acids in length. The primary amino acid sequence predicts a protein with 12 transmembrane domains in two homologous halves, each containing six transmembrane regions and two large intracytoplasmic loops encoding an ATP-binding site. P-gp consists of two homologous halves, with 43 % amino acid-sequence identity between the amino- and carboxy-terminal halves (32,33). The degree of the homology varies throughout the sequence being much stronger near the C-terminus than in the rest of the protein. Each half of P-gp consists of a short, highly hydrophilic, N-terminal region, a long hydrophobic region and a long, relatively hydrophilic cytoplasmic C-terminal region. The mass predicted from the deduced amino acid sequence is 140 kDa (34), but size fractionation by polyacrylamide gel electrophoresis indicates a size of ~170-180 kDa. This discrepancy seems due to glycosylation and phosphorylation of the P-gp (35). This general structure appears to be indicative of membrane channels and transporters (*e.g.,* in the human voltage-sensitive Na^+ channel, cystic fibrosis transmembrane conductance regulator) and is a consistent motif throughout evolution (36).

P-gp uses energy in the form of ATP to transport drugs through a channel, forrned by the transmembrane segments (37,38).

P-gp is also found generally in polarized epithelial cell layers, where it generally localizes to the apical (or luminal) surface of the cell. This localisation indicates that a major

function of P-gp is the protection of organisms against many of the toxic xenobiotics to which they can be exposed in nature (39).

Lowered extracellular pH (40), heat shock and arsenite (41), cytotoxic agents (42), transfection with oncogens (43) and with human immunodeficiency virus-1 (44) and UV irradiation (45), increase the expression of the MDR1 gene in both human and rodent cell lines.

Expression of multidrug transporter in normal tissues, the normal function of "MDR"

The highest level of human MDRl protein and mRNA expression are found in the adrenal gland, kidney, jejunum, colon and endothelial cells of the blood-brain barrier, whereas human MDR2 is strongly expressed in the liver.

Immunohistochemical analysis with specific anti-P-gp antibodies reveals that P-gps are expressed in a polarized manner on the apical membrane of secretory epithelial cells lining luminal spaces such as the glandular epithelial cells of the endometrium in the pregnant uterus, the biliary canaliculi of hepatocytes, the brush border of renal proximal tubules, pancreatic ductules, columnar epithelium of the intestine and in endothelial cells of the blood- brain barrier and in the testes (13). Expression of P-gp at the surface of epithelial cells lining the luminal spaces of the intestine, kidney and liver suggest that P-gp plays a protective role at these sites. Additional experimental evidence suggests that:

1. endothelial cells from the blood-brain barrier can carry out unidirectional drug transport *in vitro* (46) to protect the brain from toxic natural products (12);
2. pluripotent stem cells of the hemopoetic system can transport the P-gp substrate fluorescent dye rhodamine 123 (47);
3. the cardiac glycoside digoxin, which is eliminated by glomerular filtration and tubular excretion, is a P-gp substrate (48). On the other hand, expression of P-gp at other sites such as the pancreatic ductules, the adrenal cortex and the endometrial glands of the pregnant uterus, suggest that P-gp may play a role in hormone transport. P-gp can transport steroid hormones such as aldosterone, dexamethasone, cortisol (49) and corticosterone.

The tissue-specific expression of the MDR1 gene indicates that levels of MDR1 RNA may be regulated in mammals. Several studies have demonstrated increases in RNA levels for P-gp after partial hepatectomy (50,51), treatment with chemotherapeutic drugs and cytotoxic stress such as heat shock (41). These studies demonstrating increased P-gp mRNA levels in response to cell injury support a role for P-gp in protecting cells against damage by toxic products.

Glycosylation of P-glycoprotein

P-gps are post-translationally modified by glycosylation and phosphorylation. P-gp is N-glycosylated at one position on the first extracellular loop of the C-terminal. Experiments in which multidrug resistant cells were grown in the glycosylation inhibitor of tunicamycin (52,53) and mutant multidrug resistant cells greatly reduced glycosylation of P-gp demonstrating that glycosylation of P-gp is not required for its multidrug transport function (54,55). However, it has been suggested that N-glycosylation may contribute to the correct folding, proper routing and stabilization of the molecule.

Phosphorylation of P-glycoprotein

Early studies suggested that the phosphorylation state of P-gp affects its function. P-gp is phosphorylated on serine and threonine residues and is a substrate for a number of cellular kinases (56,57). Studies in which protein kinase C has been specifically overexpressed, activated by phorbol esters (58,59) or inhibited by staurosporine (60-62), have demonstrated that P-gp is a substrate for protein kinase C phosphorylation (63,64). In human P-gp, protein kinase C phosphorylation sites are Ser-661, Ser-671 (65); cAMP dependent protein kinase phosphorylation sites are Ser-667, Ser-671 and Ser-683 (66). These phosphorylation sites are confined to a central cytosolic segment that connects the two homologous halves of P-gp. It is interesting that some of the multidrug resistance reversing agents, such as verapamil and trifluoperazine, which bind and inhibit P-gp, also increase P-gp phosphorylation (67,68). These studies suggest that different kinases are involved in P-gp phosphorylation; namely, cellular drug resistance is increased which is correlated with decreased intracellular drug accumulation.

Interaction of P-glycoprotein with drug molecules

The hypothesis that P-gp protects multidrug resistant cells by pumping cytotoxic drugs through the plasma membrane out of the cell, suggests that the P-gp contains specific binding sites for these drugs and their sites could be labeled by photoaffinity drug analogs. Many different photoaffinity derivatives of multidrug resistant drugs have been synthesized and shown to bind to P-gp; the most frequently used are the drug analog 3H-vinblastine (69), the P-gp modulator calcium-channel blocker 3H-azidopine (70), verapamil (71), an α1-adrenergic receptor ligand ^{125}I-iodoarylazidoprazosin (72) and others like colchicin (73), iodomycin (74), cyclosporin A (75), and forskolin (76).

More studies have established that at least two regions of the membrane-associated portion of P-gp are involved in binding photoactivable ligands: one minor site located within the amino terminal half, and a major site mapping to the carboxy terminal half (77,78).

A further study on a series of antibodies directed against discrete P-glp peptides demonstrated that prazozine and azidopine bound to the same site on P-gp, which was limited mostly to two 4 and 5 kDa cryptic peptides mapping immediately downstream of the last membrane domains of

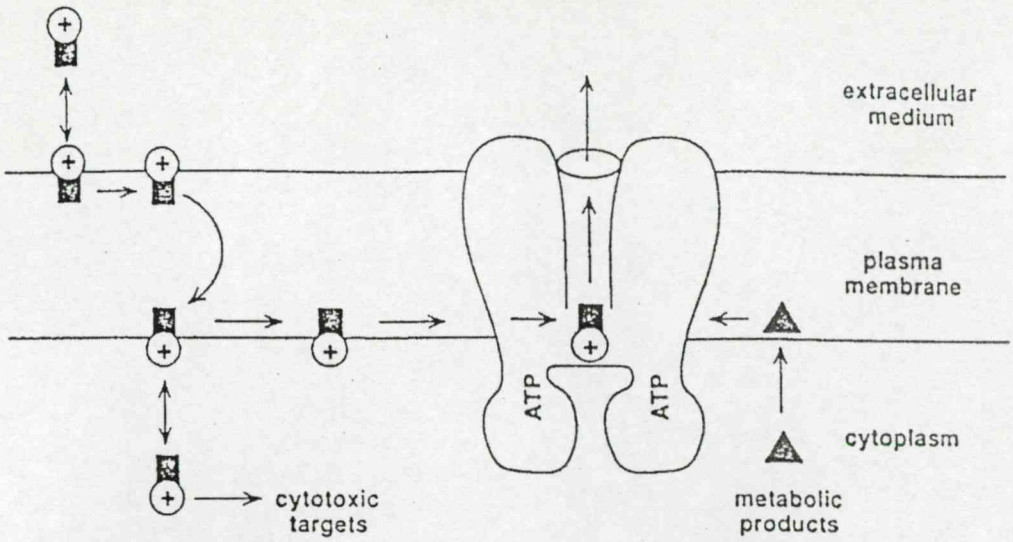


Figure 2. *Mechanism of action of the mdr efflux pump.*

each half of the P-gp (79). This study suggested that symmetrical regions in each half of P-gp are involved in drug binding and transport.

Cornwell *et al.* (80) prepared two different photoaffinity vinblastine analogs and showed that these compounds specifically labeled P-gp. The specificity of labeling was defined by three criteria: the photoaffinity vinblastine analogs labeled a 170 kD · protein in the plasma membrane of multidrug resistant cells, but did not label any such protein in the plasma membrane vesicles prepared from the drug sensitive parent cells; it was possible to inhibit the action by adding excess vinblastine to the reaction; the 170 kDa labeled protein was immunoprecipitated by antibodies raised against P-gp (81).

Excess vinblastine competed succesfully to prevent ^{3}H-vinblastin from binding to plasma membrane vesicles prepared from multidrug resistant cells. These results show that vinblastin binding, and labeling is a saturable process and from this one may infer that vinblastin binds to a specific site on P-gp. Other drugs, which are also presumed to be substrates for P-gp transport, did not inhibit vinblastin binding and labeling. For instance, daunomycin inhibited vinblastin binding or tomato lectin inhibited promethazine binding to P-gp, but colchicine and actinomycin D did not, even though the multidrug resistant cells are crossresistant to all of these drugs (38).

Many different drugs are able to interfere with the activity of the multidrug transporter and also reverse the multidrug resistance phenotype of cultured cells. These include the calcium channel blocker verapamil, the antiarrhythmic quinidine, the antihypertensive reserpine and the immune suppressant cyclosporin A (82). Since verapamil is also a substrate for P-gp (83) and inhibits ATP-dependent transport of vinblastin into vesicles containing P-gp (38), it is likely that verapamil works as a competitive inhibitor of the multidrug transporter.

Table III. *Methods of detection MDR.*

Analysis of RNA	Analysis of protein
Northern blot, slot blot	Western blot
RNAse protection	Immunohistochemistry
In situ hybridization	Flow cytometry
Polymerase chain reaction (PCR)	

Photoaffinity analogs of verapamil (84), colchicine (85) and daunomycin (74) have since been prepared and used to label specifically P-gp. The results demonstrate that these drugs have binding sites on P-gp and support the hypothesis that P-gp is responsible for pumping them out of the multidrug resistant cells. Probably all the drugs share a single site for the transport by P-gp, but there are more than one site on the transporter for binding.

Expression of multidrug transporter in human cancer

A preliminary survey of more than 400 different human cancers demonstrated the widespread expression of the MDRl gene in human cancers with both intrinsic and

Table IV. *Chemosensitizers: agents capable of inhibiting P-gp in vitro.*

Agents that reverse drug resistance	*Anticancer drugs*
Calcium channel blockers (verapamil, nifedipine, azidopine, dihydropteridines)	Vinca alkaloids (vinblastine, vincristine)
Anti-arrhythmics (quinidine, amiodarone)	Anthracyclines (doxorubicine)
Antihypertensives (reserpine)	Epipodophyllotoxins (etoposide)
Antibiotics (hydrophobic cephalosporins)	Antibiotics (actinomycin D)
Antihistamines (terfenadine)	Others (mitomycin C, taxol, mithramycin, topotecan)
Immunosuppressants (cyclosporin A, FK506, rapamycin)	*Other cytotoxic agents*
Steroid hormones (progesterone)	Antimicrotubule drugs (colchicine, podophyllotoxin)
Modified steroids (tamoxifen, tirilazad)	Protein synthesis inhibitors (puromycin, emetine)
Lipophilic cations (tetraphenylphosphoniurn)	DNA intercalators (ethidium bromide)
Diterpenes (forskolin)	Toxic peptides (valinomycin, gramicidin, D,N-acetyl-leucyl-leucyl-norleucinal (ALLN))
Detergents (Tween-80)	
Antidepressants (thioperidone)	
Antipsychotics (phenothiazines)	
Many other hydrophobic, amphipathic drugs and their analogs	

acquired multidrug resistance (86). Cancers of the liver, colon, kidney, pancreas and adrenal, which are generally drug-resistant, express high levels of MDR1 RNA equivalent to levels found in four-fold to six-fold multidrug resistant tissue culture cells (86). Similar levels of expression are found in a minority of human leukemias and non-Hodgkin's lymphoma, chronic myelogenous leukemia in blast crisis, astrocytoma before chemotherapy and in higher percentages of treated leukemias, lymphomas, neuroblastomas, sarcomas, cancer of the breast and ovary from patients who have received chemotherapy. The process of malignant transformation in cancer derived from tissues that do not normally express P-gp can activate expression of the MDRl gene (43). Increased expression of the MDRl gene is commonly seen in tumors treated with chemotherapy that have relapsed during the course of, or after chemotherapy. For example breast cancer, ovarian cancer, lymphoma, leukemia, neuroblastoma, pheochromocytoma, rhabdomyosarcoma and multiple myeloma. In these cases, it is presumed that small numbers of MDR1-expressing cells were present when therapy was initiated and that this population survived chemotherapy and caused the relapse. But a direct effect of chemotherapy to induce mdrl gene expression is also possible (87).

Mechanism of action

The early studies of P-gp-positive multidrug resistant cells clearly demonstrated that the emergence of multidrug resistance in these cells was linked to a marked decrease in the intracellular accumulation of the various drugs to which the cells expressed resistance. The reduced cellular drug accumulation was strictly energy (ATP) dependent and was concomitant with an ATP-dependent increase in drug release from these cells. Two major hypotheses for the mechanism of P-gp action to explain such reduced drug accumulation have been put forward (3 1,54,88).

The first hypothesis is that P-gp functions as a drug transporter (efflux pump) which can act on a broad range of structurally unrelated molecules and uses the energy of ATP hydrolysis to mediate drug efflux. Experimental data supporting the notion that P-gp functions as a drug transporter include the observations that: P-gp binds the ATP analog, has ATPase activity and mutations in either of its predicted ATP-binding domains to abrogate its function; P-gp binds drug analogs and mutations in its predicted transmembrane domains and modulates substrate specificity by altering drug binding to the protein; P-gp shares homology with a number of prokaryotic and eukaryotic membrane proteins implicated in the ATP-dependent transport of various types of substrates across the membrane; transport studies in intact cells and plasma membrane vesicles from P-gp expressing cells indeed suggest that P-gp mediates increased ATP-dependent drug binding and transport into these vesicles (38,80,89).

The second hypothesis proposes that P-gp itself is not a drug transporter, but has an indirect role in modifying the intracellular environment to create either an electrochemical - or a pH - gradient. In turn, these gradients would act in an indirect fashion to drive the movement of charged drugs across the membrane. The major appeal of this hypothesis is its explanation of the unusual ability of P-gp to act on a vast number of structurally unrelated substrates which yet share a high degree of hydrophobicity and are positively charged at

neutral pH. In this model, P-gp was proposed to function as an outwardly directed ATP channel, creating an electrochemical ATP-gradient and possibly providing a driving force for drug efflux. Another model for the P-gp nrechanism is based on the observation that P-gp-positive cells demonstrate an altered intracellular pH (90). In this model P-gp would function either directly or indirectly to increase intracellular pH and lower the electrical membrane potential, in consequence of which: charged hydrophobic compounds such as anticancer cytostatics or multidrug resistance reversing drugs (lipophilic cations) might be differently retained in P-gp-positive and negative cells; the pH-dependent binding of drug molecules to their respective, but structurally unrelated targets, might also be altered by P-gp overexpression.

These hypotheses suggest that the P-gp action may have a dual function.

A model for the action of P-gp, which incorporates these ideas, is shown in Figure 2. (32). The first feature of this mechanism is that drugs can be detected and expelled as they enter the plasma membrane in the manner of a hydrophobic vacuum cleaner. Anticancer drugs with a hydrophobic domain and a positively charged domain demonstrate diffusion into and across the plasma membrane, where they encounter the multidrug transporter by lateral diffusion. The transporter uses energy transduced from two essential ATP binding sites to pump the drug out of the membrane (91). The hydrophobic metabolic product, produced in the cytoplasm or in the membrane, is shown as a potential substrate for the transporter. One pathway of drug or metabolic efflux within the transporter is shown for simplicity, but there may be more than one (92).

Methods of MDR1 detection

The detection of multidrug resistance has both theoretical and clinical significance. These approaches include assessing MDRl gene amplification, measuring MDR1 mRNA levels and detecting P-gp. General methods for detecting MDR1 genes in tissues are shown in Table III.

A number of monoclonal antibodies are available for the detection of P-gp. The monoclonal antibodies C219 used for both immunoblot analysis and immunochistochemical detection of P-gp is nonspecific. Another antibody C494 is specific for P-gp. This antibody also recognizes a cytoplasmic epitope and thus requires fixation prior to immunohistochemical staining. Antibody MRK-16 has high affinity for P-gp. MRK-16 recognizes an external epitope on the outer surface of the plasma membrane of living cells and it does not require prior fixation. The primary advantages of immunohistochemistry in the clinical setting include the ability to discriminate P-gp expression in normal cells compared to tumor cells. Immunohistochemistry is sensitive enough to detect low-level expression of P-gp. Polymerase chain reaction is probably the most sensitive, rapid and easy means of detecting MDR1 mRNA. It is possible to analyze the most minute clinical specimens for diagnostic purpose.

Clinical relevance of reverse multidrug resistance

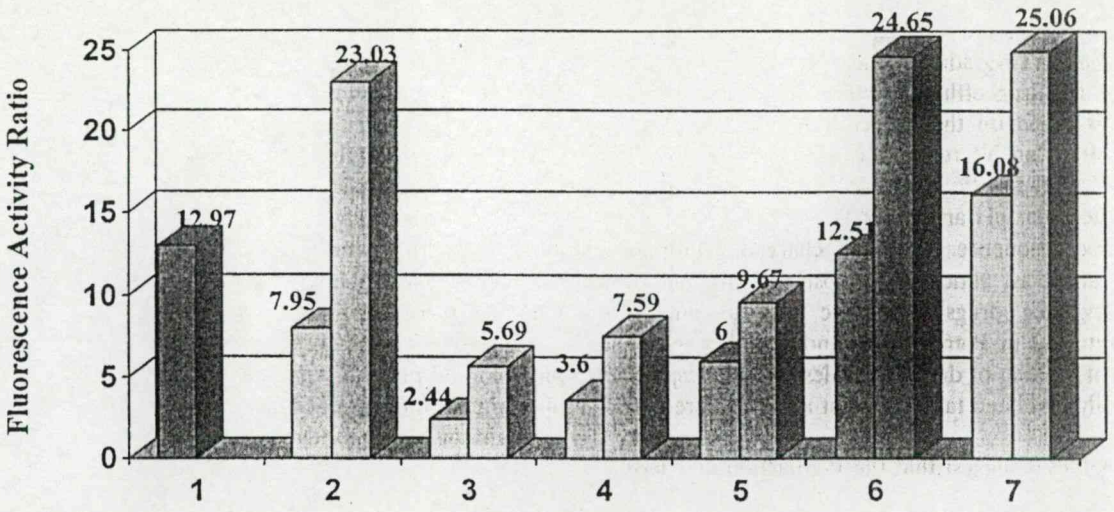
After the discovery that the P-gp molecule can be expressed in human tumors, clinical trials have been initiated with the aim to block the P-gp drug efflux pump and in that way make anticancer drugs more effective (93). Large numbers of compounds have been noted to overcome P-gp mediated multidrug resistance (Table IV).

Chemosensitizers in general are lipid-soluble at physiological pH and possess a basic nitrogen atom and two planar aromatic rings at least. The calcium channel blocker verapamil is able to overcome P-gp mediated multidrug resistance to vincristine and doxorubicin, both *in vitro* and *in vivo.* Verapamil is capable of overcoming clinical drug resistance, but cardiotoxicity has been close-limiting. Cardiotoxicity induced by verapamil may be reduced by specific stereoisomers. Both the R and S optical isomers of verapamil are equally effective in reversing multidrug resistance, but the R isomer is 10 times less cardiotoxic that the S isomer. Clinical studies are currently underway with R-verapamil as a chemosensitizer, and likewise the immunosuppressive drug cyclosporine and its non-immunosuppressive analogue SDZ PSC 833.

The role of stereoselectivity

Chiral anticancer drugs exist since pharmacological differences have been found between stereoisomers. The single isomers of leucovorin, isophosphamide buthionine sulfaximine and verapamil are used in medical practice with better results than the racemic forms, however the role of chirality was not properly analysed (94,95) in the reversal effect of drugs on the mdr. Despite similar drug targets in the cancer cells, the 50 % inhibitory concentration of various anthracyclines, vinca alkaloids, podophyllotoxins, topoisomerase inhibitors and antibiotics varied to a great extent in multidrug resistant and sensitive cancer cells (96).

In the multidrug resistant cancer cells the efflux-pump mechanism is responsible for treatment failures, therefore inhibition of the efflux mechanism may result in an effective anticancer chemotherapy. However, we have to consider that normal cells also contain ABC transporters *e.g.,* to function for detoxification. To avoid toxic side effects of novel resistance reversal compounds we need drugs with selective inhibition of the mdr efflux without any effects on ABC transporters with physiological function in cancer cells. To achieve this effect three classes of known neuroleptic drugs with active and inactive stereoisomers were tested for mdr efflux inhibition and antiproliferative effects on sensitive and multidrug resistant cancer cell cultures and the results were compared with verapamil as a classic resistance modifier. Before the reversal of multidrug resistance, the ID_{50} values of



1: Verapamil 5 μg/mL; **2:** (-)Butaclamol 0.5; 5 μg/mL; **3:** (+)Butaclamol 0,5; 5 μg/mL; **4:** L-methotrimeprazine 0.5; 5 μg/mL; **5:** D-methotrimeprazine 0.5; 5 μg/mL; **6:** Trans(E)-clopentixol 0.5; 5 μg/mL; **7:** Cis(Z)-clopenthixol 0.5; 5 μg/mL

Figure 3. *The effect of butaclamol, methotrimeprazine and clopenthixol isomers on the function of MDR efflux pump in mouse lymphoma cells.*

the compounds were determined and it was found that the parent cell line, having 30 % higher ID_{50}, was less sensitive to the stereoisomer pairs than the multidrug resistant cell line. However, we have to consider that, for the determination of antiproliferative effect, a much smaller number of cells is treated for a much longer time than for the determination of reversal of the MDR efflux-pump.

The effect of three different pairs of stereoisomers was studied on the activity of MDR-glycoprotein of mouse lymphoma cells (Figure 3.). The levo- and dextro-methotrimeprazine enantiomers had nearly the same effect by inhibiting the efflux-pump activity of mdr P-gp in mouse lymphoma cells. The results show that the two enantiomers do not differ significantly in the reversal of multidrug resistance.

The second pair was the butaclamols derivatives. An interesting relationship was found between mdr-reversing effect and molecular configuration of the butaclamol stereoisomers. In the experiments the concentration dependent inhibition of stereoisomers on the efflux pump were compared. (-)Butaclamol was more effective than the biologically important (+)butaclamol enantiomer. The data indicated that there are no general rules for structure-activity relationships in the inhibition of mdr efflux pump activity by tricyclic compounds.

The third stereoisomer pair concerns the clopenthixols. The cis- and trans-clopenthixol had similar inhibitory effects on the mdr efflux-pump of mouse lymphoma cells if results were compared to verapamil as a control.

The (-) isomer of verapamil is 10-fold more potent as a calcium antagonist than the (+) isomer, however both enantiomers were reported as equally effective in increasing cellular accumulation of anticancer drugs (97). The enantiomers of phenylalkylamines are equally potent in inhibiting drug transport by P-gp (98). However the potency of vinca stereoisomers in both wild type and mdr cells was dependent on the substituents of stereoisomeric form due to modulations of cytotoxicity of vinblastine (99). When the stereoisomers of verapamil and cyclosporins were compared on P-gp mediated efflux and NK cell mediated cytolysis, the verapamils were more potent inhibitors of cytolysis than the cyclosporin. On the contrary, cyclosporins were more effective for inhibiting P-gp mediated rhodamine efflux than the verapamil isomers (100).

The CNS active and inactive members of butaclamol enantiomers differed in MDR-reversal very much, that means that the drug binding has some enantioselectivity on the P-gp. On the other hand, the similar effects of levo- and dextromethotrimeprazine provide evidence against the enantioselective inhibition of the drug efflux pump.

How can the two different examples of enantiomer effects be exploited ? The CNS inactive enantiomers were inhibitory in the case of methotrimeprazine and butaclamol. Therefore, the mdr reversing effects of CNS inactive members of enantiomers can he exploited in neoadjuvant chemotherapy of cancer to increase the effectiveness of several cytostatics in multidrug resistant cancer. The remarkable inhibitory effect

of (-)butaclamol may be due to the higher log-P value, *i.e.,* the increased lipophilicity.

Surprisingly, the stable stereoisomers of clopenthixol had similar concentration-dependent inhibition on the mdr efflux mechanism; again evidence for the lack of stereospecificity in drug binding of cancer cells (101). On the contrary, the normal cells in the CNS (102), in the heart (97), bacterial plasmid replication inhibition of ATPase or cholinesterase enzymes (103), stereoselectivity and configuration of tricyclic compounds is essential (102).

Cis-clopenthixol inhibited the dopamine stimulated adenyl cyclase 10-fold compared to the trans isomer. In the case of methotrimeprazine enantiomers a significant stereoselective effect was observed on dopamine and 5-hydroxytryptamine receptors, whereas the opiate receptors did not discriminate between the levo- and dextrorotatory isomers (104).

The basis of selectivity of some stereoisomers may be due to a rigid configuration of the receptor sites. Another concept is that the individual members of stereoisomer pairs have different energy levels for binding. Indeed, the cis-stereoisomer has a more stable configuration with a lower energy level than the trans-form of clopenthixol. The differences in the energy levels in the excited state can be even higher (105).

Based on the relatively high concentration of drugs used in our experiments, potential non-specific effects was suggested *via* interactions at various drug receptors. As an example, sigma receptors have high affinity binding sites for several psychotropic drugs. Similar sites are located not only in the CNS but also occur in various peripheral tissues. The overexpression of these receptors is found in human tumors (106). Different neuroleptic compounds affecting sigma receptors produced changes in cell morphology. Some neuroleptics lacking sigma affinity such as (+)butaclamol and clozapine had no effect on cellular morphology (107). However, (-)butaclarnol exhibited morphological changes resembling apoptosis. The binding sites are distinguishable by their affinity for stereospecific ligands (108).

The (+)butaclamol has a high affinity binding site on the dopamine D2 receptor (109) while the (-)butaclamol was 30 times less active. Interestingly, the (-)enantiomer was more active in the reversal of the MDR efflux-pump than the (+)butaclamol, which means that the MDR reversal effect was not mediated by D2-like structures, but probably mediated by sigma receptors. 5-Hydroxytryptamine receptors are suggested to play a role in certain neuroleptic disorders; the therapeutic effect of (+)butaclamol and clozapine is localized on 5HT receptors by inhibiting some effects of 5HT. However the (-)butaclamol and (-)propranolol were less effective (1, 10).

Considering the role of D4 receptors or D~-like structures on the mdr glycoprotein, we can exclude D4 specific binding on the P-gp because doparnine receptor antagonists showed a high affinity to the receptor with a rank order of haloperidol > chlorpromazine > (+)butaclamol > (-)butaclarnol. The (+)butaclamol bound to D_4 receptors in a stereoselective manner, showing higher affinity than its respective (-) enantiomer. The (+) enantiomer was found to be nearly 20-fold more effective than (-)butaclamol (111). The lack o,f dopamine receptor specific stereospecificity of mdr-reversal excludes the involvement of D_2 and D_4-like binding sites on the P-gp; rather our results refer to the sigma receptor involvement for drug binding responsible for inhibition of drug efflux in the tumor cells.

When mdr cells were exposed to tricyclic stereoisomers with lipophilic characteristics the drug resistance was reversed due to the inhibition of the efflux-pump system. However, down-regulation of the mdr gene was also found in some cases (112,113). It was interesting that the effect of verapamil stereoisomers had no substantial difference in the potencies of (+) and (-) enantiomers in reversing the mdr efflux pump (98), and in accelerating clearance (114).

The enantioselective mdr-reversal effect of the pharmacologically inactive (-)butaclamol may be exploited in combination chemotherapy based on the different lipophilicity of the two stereoisomers.

Table V. *Influence of anti-psychotic drugs on the uptake of daunorubicin, as P-gp substrate, into PBL of patients.*

Antipsychotic drugs	Conc. μg/ml	Relative fluorescence intensity		
		Patients: SzL	PL	NV
Amitriptiline	1	-	-	-
	5	1.48	1.39	1.10
Fluphenazine	1	-	-	-
	5	1.24	1.53	0.90
Maprotiline	1	-	-	-
	5	1.86	1.32	1.03
Trimipramine	1	-	-	-
	5	1.53	-	-
Desipramine	1	-	-	0.90
	5	1.65	-	-
Imipramine	1	-	-	-
	5	1.42	1.00	1.00
Haloperidol	1	-	-	-
	5	1.44	1.37	0.98
Cyclosporin A	5	1.39	1.32	0.90

Anti-psychotic drugs reverse multidrug resistance of tumor cell lines and human AML cells *ex vivo*

Several compounds are in clinical trials for modulation of P-gp related resistance in cancer (27). It is now not uncommon to use antipsychotic drugs in cancer patients (115,117). The concomitantly used cancer chemotherapeutic and anti-psychotic drugs may alter each others pharmacokinetics in cancer patients. If the anti-psychotic drug also affects P-gp function then the pharmacokinetics of the anticancer drugs may change in cancer cells, and also pass through the blood-brain barrier (118).

Tricyclic antipsychotic drugs were shown to exert a variety of biological effects at the subcellular and cellular levels. For example, chlorpromazine (CPZ), trifluoperazine and clozapine inhibit certain proteases, acetylcholine esterase and affect Ca^{2+} metabolism (119).

Fluphenazine, a phenothiazine type antipsychotic drug, some of its analogues (120), butaclamol stereoisomers (101) and the chemically related thioxanthene type compounds (121), were shown to inhibit P-gp function in some MDRI gene expressing cell lines. Most of these tricyclic- and other antidepressive drug molecules are lipophilic and possess a positive charge due to their terminal chain nitrogen atom. These chemical characteristics were shown to be important for agents which affect the function of P-gp (14,31). Prochlorperazine, the tricyclic antiemetic drug, was shown to be effective as a resistance modifier in phase I clinical studies (122).

For the above reasons we studied the effect of some antipsychotic drugs on the function of P-gp. In our study, we used the transfected leukemia cell lines L1210 MDR, L5178 MDR and the human adenocarcinoma cell line KB-V-1 and peripheral blood lymphocytes obtained from leukemic patients. Treatment of L1210 MDR cells with cyclosporin A (CsA), (5 μg/mL), a known P-gp blocker, increased the relative fluorescence intensity about 7-fold in the L1210 MDR cells. All tested antipsychotic drugs increased the relative fluorescence intensity in L1210 MDR cells, haloperidol and fluphenazine being the most active. In the L5178 MDR cell line fluphenazine and maprotiline were the most effective compounds. None of the tested drugs changed significantly the rhodamine 123 uptake into the parental cells. Similar results were obtained with KB cells when daunorubicin (DR) was used as a fluorescent substrate (123).

In these experiments DR was used as fluorescent substrate of P-gp, since it was also used as chemotherapeutic agent in patients. The antipsychotic drugs increased the relative fluorescence of KB-V-1 but not of the KB-3-1 parental cells. The increase was close to that with CsA, the known P-gp blocker, for amitriptyline, fluphenazine and haloperidol. However, efflux of DR from KB-V-1 cells was not blocked to the same extent as in the L1210 MDR and L5178 MDR cells relative to CsA. We attributed this lack of correlation between the three cell lines to the fact that we used different substrates, rhodamine 123 and DR with the L5178, L1210 and the KB cells, respectively, which fact can contribute to differential sensitization with CsA. It was shown previously that some P-gp blockers may exert their effects indirectly through the plasma membrane without being a substrate (124). Because of the demonstrated blocking effect of the tested antipsychotic drugs on P-gp, these drugs were tested in peripheral blood lymphocytes (PBL) of drug treatment resistant patients, using DR as fluorescent substrate. Relative to L1210 MDR, L5178MDR and to KB-V-1 cells, CsA had a small effect on DR efflux in PBL of the patients SzL and PL. However, most of the tested antipsychotic drugs had comparable effects to CsA in PBL in both of the patients (Table V).

Cells from acute myeloid leukemia (AML) patient NV, although resistant to treatment in the clinic, could not be sensitized by CsA or with the antipsychotic drugs for uptake of DR (Table V). The reason for the difference among the three PBL for sensitization could not be established. However, some basic information can be obtained for example by mAb staining. Future investigations are planned to include more detailed characterisation of clinically resistant AML cells, which do not respond to P-gp blockers.

Antipsychotic drugs are used at blood levels of 10 to 500 ng/mL, depending on the drug used and on the sensitivity of the patient (121,125). A double concentration of the highest clinical blood level, 1 μg/mL, showed small but significant blocking of P-gp in L1210 MDR, L5178 MDR and KB-V-1 cells. PBL of the patients could not be tested at a 1 μg/mL dose level, due to an insufficient amount of blood samples. However, based on results with CsA at a comparable dose level, 5 μg/mL, it was expected that uptake of DR into PBL would be enhanced by 1 and 0.5 μg/mL of some of the antipsychotic drugs.

Taken together, the results obtained with the use of the model cell lines L1210 MDR, L5178 MDR and KB-V-1 and the fluorescent indicator R 123 and DR, we showed that the clinical anti-psychotic drugs may significantly modulate the uptake of substrates of P-gp into MDR cells. We have also tested this possibility in a pilot study, using PBL prepared from the blood of drug resistant leukemic patients. Resistance was based on clinical experience and was verified on PBL by the effect of CsA on the uptake of DR. In these PBL cells we compared the effects of the antipsychotic drugs with that of CsA for the uptake of DR, the drug used for the treatment of two of the patients. The *ex vivo* results indicated that treatment with some of the antipsychotic drugs changed as much in DR uptake as CsA does in two out of three patients' PBL. Our results point in the direction of necessary pharmacological studies when antipsychotic drugs and cancer chemotherapeutic agents are used together in multidrug resistant cancer patients.

Other possibilities may exist to overcome multidrug resistant malignancies such as the administration of differentiation inducers, apoptosis inducers, vaccination against the tumor cells or some thermodynamic approaches.

References

1 Motohashi N, Kurihara T, Kawase M, Hever A, Tanaka M, Szabó D, Nacsa J, Yamanaka W, Kerim A and Molnár J: Drug resistance reversal, anti-mutagenicity and antiretroviral effect of phthalimido- and chloroethyl-phenothiazines. Anticancer Res *17:* 3537-3544, 1997.

2 O'Hare K, Murphy C, Levis R and Rubin GM: DNA sequence of the white locus of *Drosophila melanogaster.* J Mol Biol *180:* 437-455, 1984.

3 Wilson CM, Serrano AE, Wasley A, Bogenshutz MP, Shankar AH and Wirth DF: Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum.* Science *244:* 1184-1186, 1989.

4 Foote SJ, Kyle DE, Martin RK, Oduola AMJ, Forsyth K, Kemp DJ and Cowman AF: Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum.* Nature *345:* 255-258, 1990.

5 Kuchler K, Steme RE and Thorner J: *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. EMBO J *8:* 3973-3984, 1989.

6 McGrath JP and Varshavsky A: The yeast STE6 gene encodes a homologue of the mamrnalian multidrug resistance P-glycoprotein. Nature *340:* 400-4, 1989.

7 Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N and Chou JL: Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science *245:* 1066- 1073, 1989.

8 Hyde SC, Emsley P, Hartshorn MJ, Mimrnack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE and Higgins CF: Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature *346:* 362-65, 1990.

9 Kamijo K, Taketani S, Yokota S, Osumi T and Hashimoto T: The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. J Biol Chem *265:* 4534-4540, 1990.

10 Gartner J, Moser H and Valle D: Mutations in the 70K peroxisomal membrane protein gene in Zellweger syndrome. Nature Genet *1:* 16-23, 1992.

11 Monaco JJ: A molecular model of MHC class-I-restricted antigen processing. Immunol Today *13:* 173-79, 1992.

12 Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR: Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barriers sites. Proc Natl Acad Sci USA *86:* 695-698, 1989.

13 Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham C: Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA *84:* 7735-7738, 1987.

14 Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT and Bates SE: Rhodamin efflux patterns predict P-glycoprotein substrates in the national cancer institute drug screen. Molec Pharmacol *46:* 627-638, 1994.

15 Beck WT: Unknotting the complexities of multidrug resistance: the involvement of DNA topoisomerases in drug action and resistance. JNatl Cancer Inst *81:* 1683, 1989.

16 Fernandes DJ, Danks MK and Beck WT: Decreased nuclear matrix DNA topoisomerase II in human leukemia cells resistant to VM-26 and m-AMSA. Biochemistry *29:* 4235-4241, 1990.

17 Morrow CS and Cowan KH: Glutathione S-transferases and drug resistance. Cancer Cells *2:* 15-22, 1990.

18 Ogawa J, Iwazaki M, Inoue H, Koide S and Shohtsu A: Immunohistochemical study of glutathione-related enzymes and proliferative antigens in lung cancer. Relation to cisplatin sensitivity. Cancer *71:* 2204-2209, 1993.

19 Cole SP, Chanda ER, Dicke FP, Gerlach JH and Mirski SE: Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. CancerRes *51:* 3345-3352, 1991.

20 Kruh GD, Chan A, Myers K, Gaughan K, Miki T and Aaronson SA: Expression complementary DNA library transfer establishes mrp as a multidrug resistance gene. Cancer Res *54:* 1649-1652, 1994.

21 Deuchars KL, Duthie M and Ling V: Identification of distinct P-glycoprotein gene sequences in rat. Biochim Biophys Acta *1130:* 157- 165, 1992.

22 Ng WF, Sarangi F, Zastawny RL, Veinot-Drebot L and Ling V: Identification of members of the P-glycoprotein multigene family. Mol Cell Biol *9:* 1224-1232, 1989.

23 Chen C, Chin J, Ueda K, Clark I, Pastan I, Gottesman MM and Roninson I: Internal duplication and homology with bacterial transport proteins in the mdrl (P-glycoprotein) gene from multidrug-resistant human cells. Cell *47:* 381-389, 1986.

24 Chen C, Clark D, Ueda K, Pastan I, Gottesman MM and Roninson I: Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. J Biol Chem *265(1):* 506-514, 1990.

25 Callen D, Baker E, Simmers R, Seshadri R and Roninson I: Localization of the human multiple drug resistance gene, MDR1, to 7q21.1. Hum Genet *77:* 122-126, 1987.

26 Chin JE, Soffir R, Noonan KE, Choi K and Roninson IB: Structure and expression of the human MDR (P-glycoprotein) gene family. Mol Cell Biol *9(9):* 3808-3820, 1989.

27 Dicato M, Duhem C, Pauly M and Reis F: Multidrug resistance: molecular and clinical aspects. Cytokines Cell Mol Ther *3(2):* 91-100, 1997.

28 Nooter K and Stoter G: Molecular mechanisms of multidrug resistance in cancer chemotherapy. Path Res Pract *192:* 768-780, 1996.

29 Azzaria M, Schurr E and Gros P: Discrete mutations introduced in the predicted nucleotide- binding sites of the mdrl gene abolish its ability to confer multidrug resistance. Mol Cell Biol *9:* 5289-97, 1989.

30 Dhir R and Gros P: Functional analysis of chimeric proteins constructed by exchanging homologous domains of 2 P-glycoproteins conferring distinct drug resistance profiles. Biochemistry *31:* 6103-6110, 1992.

31 Gottesman MM and Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem *62:* 385-427, 1993.

32 Gottesman MM, Schoenlein PV, Currier SJ, Bruggemann EP and Pastan I: Biochemical basis for multidrug resistance in cancer. Biochemical and Molecular Aspects of Selected Cancer, Vol.1, Chapter 11, 1991.

33 Kane SE, Pastan I and Gottesman MM: Genetic basis of multidrug resistance of turnor cells. J Bioenerg Biomembr *22(4):* 593-618, 1990.

34 Beck WT, Cirtain MC and Lefko JL: Energy-dependent reduced drug binding as a mechanism of Vinca alkaloid resistance in human

leukemic lymphoblasts. Mol Pharmacol *24:* 485-492, 1983.
35 Futscher BW and Dalton WS: P-glycoprotein mediated multidrug resistance, Drug Resistance in Oncology. Edited by B. A. Teicher. chapter *19:* 461-479, 1993.
36 Catterall WA: Structure and function of voltage-sensitive ion channels. Science *242:* 50-61, 1988.
37 Hamada H and Tsuruo T: Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. Cancer Res *48:* 4926-4932, 1988.
38 Horio M, Gottesman MM and Pastan I: ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci USA *85:* 3580-3584, 1988.
39 Borst P and Schinkel AH: What have we learnt thus far from mice with disrupted P-glycoprotein genes ? Eur J Cancer *32A:* 985-990, 1996.
40 Wei LY and Roepe PD: Low external pH and osmotic shock increase the expression of human MDR protein. Biochemistry *33:* 7229-7238, 1994.
41 Chin KV, Tanaka S, Darlington G, Pastan I and Gottesman MM: Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. J Biol Chem *265(1):* 221-226, 1990.
42 Chaudhary PM and Roninson IB: Induction of multidrug resistance in hurnan cells by transient exposure to different chemotherapeutic drugs. J Natl Cancer Inst *85:* 632-639, 1993.
43 Chin KV, Ueda K, Pastan I and Gottesman MM: Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. Science *255:* 459-462, 1992.
44 Gollapudi S and Gupta S: Human immunodeficiency virus-l induced expression of P- glycoprotein. Biochem Biophys Res Commun *171:* 1002-1007, 1990.
45 Uchiurni T, Kohno K, Tanimura H, Matsuo K, Sato S, Uchida Y and Kuwano M: Enhanced expression of the hurnan multidrug resistance 1 gene in response to UV light irradiation. Cell Growth Differ *4:* 147-157, 1993.
46 Tatsuta T, Naito M, Mikami K and Tsuruo T: Enhanced expression by the brain matrix of P-glycoprotein in brain capillary endothelial cells. Cell Growth Differ *5(10):* 1145- 1152, 1994.
47 Chaudhary PM and Roninson IB: Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell *66(1):* 85-94, 1991.
48 Tanigawara Y, Okarnura N, Hirai M, Yasuhara M, Ueda K, Kioka N, Komano T and Hori R: Transport of digoxin by hurnan P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PKl). J Pharmacol Exp Ther *263(2):* 840-845, 1992.
49 Ueda K, Okarnura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T and Hori R: Human P-glycoprotein transports cortisol, aldosterone, and dexarnethasone, but not progesterone. J Biol Chem *267(34):* 24248-24252, 1992.
50 Marino PA, Gottesman MM and Pastan I: Regulation of the multidrug resistance gene in regenerating rat liver. Cell Growth Differ *1(2):* 57-62, 1990.
51 Thorgeirsson SS, Huber BE, Sorrell S, Fojo A, Pastan I and Gottesman MM: Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. Science *236(4805):* 1120-1122, 1987.
52 Beck WT and Cirtain M: Continued expression of Vinca alkaloid resistance by CCFR-CEM cells after treatment with tunicamycin or pronase. Cancer Res 42: 184-189, 1982.
53 Ichikawa M, Yoshimura A, Furukawa T, Sumizawa T, Nakazima Y and Akiyama SI: Glycosylation of P-glycoprotein in a multidrug-resistant KB cell line and in human tissues. Biochim Biophys Acta 1073: 309-315, 1991.
54 Endicott JA and Ling V: The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem *58:* 137-171, 1989.
55 Schinkel A, Kemp S, Dolle M, Rudenko G and Wagenaar E: N-glycosylation and deletion mutants ofthe human MDR1 P-glycoprotein. J Biol Chem *268:* 7474-7481, 1993.
56 Mellado W and Horwitz SB: Phosphorylation of the multidrug resistance associated glycoprotein. Biochemistry *26:* 6900-6904, 1987.
57 Roy SN and Horwitz SB: A phosphoglycoprotein associated with taxol resistance in J774.2 cells. Cancer Res *45:* 3856-3863, 1985.
58 Chambers TC, Zheng B and Kuo JF: Regulation by phorbol ester and protein kinase C inhibitors, and by a protein phosphatase inhibitor (okadaic acid), of P-glycoprotein phosphorllation and relationship to drug accumulation in multidrug resistant human-KB cells. Mol Pharmacol *41:* 1008-1015, 1992.
59 Aftab DT, Yang JM and Hait WN: Functional role of phosphorylation of the multidrug transporter (P-glycoprotein) by protein kinase-C in multidrug resistant MCF-7 cells. Oncol Res *6:* 59-70, 1994.
60 Sato W, Yusa K, Naito M and Tsuruo T: Staurosporine, a potent inhibitor of C-kinase, enhances drug accumulation in multidrug-resistant cells. Biochem Biophys Res Commun *173(3):* 1252-7, 1990.
61 Bates SE, Lee JS, Dickstein B, Spolyar M and Fojo AT: Differential modulation of P- glycoprotein trasport by protein kinase inhibition. Biochemostry *32:* 9156-9164, 1993.
62 Ma L, Marquard D, Takemoto L and Center MS: Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. J Biol Chem *266:* 5593-5599, 1991.
63 Yu G, Ahrnad S, Aquino A and Fairchild CR: Transfection with protein kinase C alpha confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. Cancer Commun *3:* 181-188, 1991.
64 Chambers TC, Chalikonda I and Eilon G: Correlation of protein kinase C translocation, P- glycoprotein phosphorylation and reduced drug accumulation in multidrug resistant human KB cells. Biochem Biophys Res Commun *169(1):* 253-259, 1990.
65 Chambers TC, Pohl J, Raynor RL and Kuo JF: Identification of specific sites in human P- glycoprotein phosphorylated by protein kinase C. J Biol Chem *268(7):* 4592-4595, 1993.
66 Chambers TC, Pohl J, Glass DB and Kuo JF: Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochem J *299:* 309-315, 1994.
67 Hamada H, Hagiwara K, Nakajima T and Tsuruo T: Phosphorylation of the Mr 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: effects of verapamil, trifluoperazine, and phorbol esters. Cancer Res *47:* 2860-2865, 1987.
68 Germann UA, Chambers TC, Ambudkar SV, Pastan I. and Gottesman MM: Effects of phosphorylation of P-glycoprotein on multidrug resistance. J Bioenerg Biomembr *27(1):* 53-61, 1995.
69 Cornwell MM, GoKesman MM and Pastan I: Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. J Biol Chem *262:* 7921-7928, 1986.
70 Safa AR, Glover CJ, Sewell JL, Meyers MB, Biedler JL and Felsted RL: Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calciurn channel blockers. J Biol Chem *262:* 7884-7888, 1987.
71 Safa AR: Photoaffinity labeling of the multidrug resistance-related

P-glycoprotein with photoactive analogs of verapamil. Proc Natl Acad Sci USA *85:* 7187-7191, 1988.

72 Greenberger LM, Yang CP, Gindin E and Horwhitz SB: Photoaffinity probes for the alpha 1-adrenergic receptor and the calcium channel bind to a common domain in P- glycoprotein. J Biol Chem *265(8):* 4394-4401, 1990.

73 Safa AR, Stern RK, Choi K, Agresti M, Tamai I, Mehta ND and Roninson IB: Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185---Val-185 substitution in P-glycoprotein. Proc Natl Acad Sci US A *87:* 7225-7229, 1990.

74 Busche R, Tummler B, Riordan JR and Cano-Gauci DF: Preparation and utility of a radioiodinated analogue of daunomycin in the study of multidrug resistance. Mol Pharmacol *35:* 414-21, 1989.

75 Foxwell BM, Mackie A, Ling V and Ryffel B: Identification of the multidrug resistance- related P-glycoprotein as a cyclosporine binding protein. Mol Pharmacol *36:* 543- 546, 1989.

76 Morris DI, Speicher LA, Ruoho AE, Tew KD and Seamon KB: Interaction of forskolin with the P-glycoprotein multidrug transporter. Biochemistry *30:* 8371-8379, 1991.

77 Bruggemann EP, Currier SJ, Gottesman MM and Pastan I: Characterization of the azidopine and vinblastine binding site of P-glycoprotein. J Biol Chem *267:* 21020- 21026, 1992.

78 Yoshimura A, Kuwazuru Y, Sumizawa T, Ichikawa M, Ikeda S, Uda T and Akiyama S: Cytoplasmic orientation and two-domain structure of the multidrug transporter, P- glycoprotein, demonstrated with sequence-specific antibodies. J Biol Chem *264(27):* 16282-16291, 1989.

79 Greenberger LM: Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. J Biol Chem *268(15):* 11417-11425, 1993.

80 Cornwell MM, Safa AR, Felsted RL, Gottesman MM and Pastan I: Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. Proc Natl Acad Sci USA *83(11):* 3847-3850, 1986.

81 Safa AR, Glover CJ, Meyers MB, Biedler JL and Felsted RL: Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. J Biol Chem *261:* 6137-6140, 1986.

82 Tsuruo T: Mechanisms of multidrug resistance and implications for therapy. Jpn J Cancer Res *79:* 285-296, 1988.

83 Cano-Gauci DF and Riordan JR: Action of calcium antagonists on multidrug resistant cells. Specific cytotoxicity independent of increased cancer drug accumulation. Biochem Pharmacol *36:* 2115-2123, 1987.

84 Qian XD and Beck WT: Binding of an optically pure photoaffinity analogue of verapamil, LU-49888, to P-glycoprotein from multidrug-resistant human leukemic cell lines. Cancer Res *50:* 1132-1137, 1990.

85 Safa AR, Mehta ND and Agresti M: Photoaffinity labeling of P-glycoprotein in multidrug resistant cells with photoactive analogs of colchicine. Biochem Biophys Res Commun *162:* 1402-1408, 1989.

86 Goldstein LJ, Galski H, Fojo AT, Willingham MC and Lai SL: Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst *81:* 116-124, 1989.

87 Bell DR, Gerlach JH, Kartner N, Buick RN and Ling V: Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. J Clin Oncol *3:* 311-315, 1985.

88 Shustik C, Dalton W and Gros P: P-glycoprotein - mediated MDR in tumor cells: biochemistry, clinical relevance and modulation. Molecular Aspects of Medicine Vol 16, No 1, chapter *1:* 5-20, 1995.

89 Kamimoto Y, Gatmaitan Z, Hsu J and Arias IM: The function of Gpl70, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. J Biol Chem *264(20):* 11693-8, 1989.

90 Roepe PD, Wei LY, Cruz J and Carlson D: Lower electrical membrane potential and altered pHi homeostasis in multidrug-resistant (MDR) cells: further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein. Biochemistry *32:* 11042-11056, 1993.

91 Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM and Pastan I: Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci USA *89:* 8472-76, 1992.

92 Kessel D: Exploring multidrug resistance using rhodamine 123. Cancer Commun *1(3):* 145-149, 1989.

93 Raderer M and Scheithauer W: Clinical trials of agents that reverse multidrug resistance. Cancer *72:* 3553-3563, 1993.

94 Wainer IW and Granvil CP: Stereoselective separations of chiral anticancer drugs and their application to pharmacodynamic and pharmacokinetic studies. Ther Drug Monit *15:* 570-575, 1993.

95 Wainer IW: Stereoisomers in clinical oncology: why it is important to know what the right and left hands are doing. Ann Oncol *2:* 7-13, 1993.

96 Isobe H, Krishan A, Wellham L and u Kawakami: Drug resitance and p-glycoprotein expression, DNA topoisomerase II activity and glutatione content in malignant mesothelioma cell lines establised after in vivo chemotherapy. in The Mechanism and New Approach on Drug Resistance of cancer cells. T. Miyazaki, F. Takaka and K. Sakurada, Elsevier Science Publishers p. 103-114, 1993.

97 Gruber A, Peterson C and Reizenstein P: D-verapamil and L-veraparnil are equally effective in increasing vincristine accumulation in leukemic cells *in vitro.* Int J Cancer *41:* 224-226, 1988.

98 Hoolt V, Kouba M, Dietel M and Vogt G: Stereoisomers of calcium antagonists which differ markedly in their potencies as calcium blockers are equally effective in modulating drug transport by P-glycoprotein. Biochem Pharmacol *43(12):* 2601- 2608, 1992.

99 Bormann LS, Bornmann WG and Kuehne ME: Modulation of drug cytotoxicity by stereoisomeric series of C-20 vinblastine congmers that lack antimicrotubule activity. Cancer Chemother Pharmacol *31/5:* 343-349, 1993.

100 Klimecki WT, Taylor CW and Dalton WS: Inhibition of cell-mediated cytolysis and P- glycoprotein function in natural killer cells by verapamil isomers and cyclosporine A analogs. J Clin Immunol *15(3):* 152-158, 1995.

101 Szabó D and Molnar J: The role of stereoselectivity of chemosensitizers in the reversal of multidrug resistance of mouse lymphoma cells. Anticancer Res *18:* 3039-3044, 1998.

102 Hyttel J: Effects of neuroleptics on ^{3}H-haloperidol and ^{3}H-cis-Z-flupenthixol binding and on adenylate cyclase activity *in vitro.* Life Sciences *23:* 551-556, 1978.

103 Molnár J, Galfi M, Lozsa A and Nakamura MJ: Inhibition of bacterial plasmid replication by stereoselective binding by tricyclic psychopharmacons. Research Comm Chem Pathol Pharmacology *43(2):* 235-249, 1984.

104 Robert TA, Hagardorn AW and Daigneault EA: Differential stereoselectivity of metotrimiprasine enantiomers for selected central nervous system receptor types. Molec Pharmacol *21:* 315-319, 1982.

105 Cumdell RB and Gilbert A: Cis-trans isomerism, In. Nelson T. ed,

Photochemistry Studies in Modern Chemistry. Academic Press, New York, N.Y. P.168, 1970.

106 Bean WT, Thomas GE, Mamone JY, Homan SM, Levy BK, Johanson FE and Coscia CJ: Overexpression of sigma receptors in non-neural human tumors. Cancer Res *51:* 6558-62, 1991.

107 Vilner BJ, de Costa BR and Bowen WD: Cytotoxic effects of sigma ligands: sigma receptor-mediated alterations in cellular morphology and viability. J Neuroscience 15/1: 117-134, 1995.

108 Hellewell SB and Bowen WD: A sigma-like binding site rat pheochromocytoma PC12 cells. Brain Res *527:* 244-253, 1990.

109 Largent BL, Wilkstrom H, Gundlach AL, Snyder SH: Structural determinants of sigma receptor affinity. J Mol Pharmacol *32:* 772-784, 1987.

110 Plassat JL, Amlaiky N and Hen R: Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Molecular Pharmacology *44:* 229-236, 1993.

111 Patel S, Marwood R, Emms F, Marston D, Leeson PD, Curtis NR, Kulagowski JJ and Freedman SB: Identification and Parmacological characterisation of ^{125}I -L-750. Molecular Pharmacology *50:* 1658-1664, 1996.

112 Molnár J, Szabó D, Mandi Y, Mucsi I, Fischer J, Varga A, Konig S and Motohashi N: Multidrug resistance reversal in mouse lymphoma cells by heterocyclic compounds. Anticancer Res *18:* 3033-3038, 1998.

113 Hever A, Santelli-Rouvier C, Brouant P, Khyari S, Molnár J, Barra Y and Barbe J: Effect of new thioacridine derivatives on p-gp function and on mdrl gene expression. Anticancer Res *18:* 3053-3058, 1998.

114 Schwartz JB, Capili H and Wainer IW: Verapamil stereoisomers during racemic verapamil administration: effects of aging and comparisons to administration of individual stereoisomers. Clin Pharmacol Ther *56(4):* 368-376, 1994.

115 Cole RM, Robinson F, Harvey L, Trethowan K and Murdoch V: Successfu'l control of intractable nausea and vomiting requiring combined ondansetron and haloperidol in a patient with advanced cancer. J Pain Symptom Manege *9:* 48-50, 1994.

116 Stiefel F and Holland J: Delirium in cancer patients. Int Psychogeriatr *3:* 333-336, 1991.

117 Vecht CJ, Hoff AM, Kansen PJ, de-Boer MF and Bosch DA: Types and causes of pain in cancer of the head and neck. Cancer *70:* 178-184, 1992.

118 Schinkel AH, Wagenaar E, Mol CAAM and van Deemter L: P-glycoprotein in the blood- brain barrier of mice influences the brain penetration and pharrnacological activity of many drugs. J Clin Invest *97:* 2517-2524, 1996.

119 Molnar J, Sohar I, Kovacs J, Rakonczay Z and Rausch H: Effect of tricyclic compounds on membrane binding of bivalent cations, activities of acetylcholinesterase and some tissue proteases. *In Vivo 7:* 431-434, 1993.

120 Ford JM, Prozialeck WC and Hait WN: Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. Mol Pharmacol *35:* 105-115, 1989.

121 Glassman AH and Perel JM: Tricyclic blood levels and clinical outcome: a review of the art. *In:* Psychopharmacology, a Generation of Progress. (Lipton AM, DiMascio A and Killam KF, eds.) Raven, NY. pp 917-921, 1978.

122 Sridhar KS, Krishan A, Samy TSA, Duncan RC, Sauerteig A, McPhee GV, Auguste ME and Benedetto PW: Phase I and pharmacokinetics studies of prochlorperazine 2-h i.v. infusion as a doxorubicin-efflux blocker. Cancer Chemother Pharmacol *34:* 377-384, 1994.

123 Szabó D, Szabó G, Ocsovszki I, Aszalos A and Molnar J: Anti-psychotic drugs reverse multidrug resistance of tumor cell lines and human AML cells *ex-vivo.* Cancer Letters *139/1:* 115-119, 1999.

124 Aszalos A, Pine PS, Pandey R and Gottesman MM: Behavior of N-acylated daunorubicins in MDR1 gene transfected and parental cells. Biochem Pharmacol *50:* 889-892, 1995.

125 Baldessarini RJ, Cohen BM and Teicher MH: Significance of neuroleptic dose and plasma level in the pharmacologic treatment of psychoses. Arch Gen Psychiatry *45:* 79- 91, 1988.

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Effects of Naturally Occurring Glucosides, Solasodine Glucosides, Ginsenosides and Parishin Derivatives on Multidrug Resistance of Lymphoma Cells and Leukocyte Funtions

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Abstract. *Solamargine, solasonine, ginsenosides and parishin-related compounds were investigated for their effects on mdr efflux pump of lymphoma cells, and their effects on T cell proliferative assays and cell mediated immune functions, antibody-dependent cellular cytotoxicity (ADCC) and natural killer (NK) cell activity of human peripheral mononuclear cells. Solamargine and solasonine were the only drugs which inhibited all of the tested immune functions; however, ginsenoside Rc and Rd enhanced T cell proliferative assays and marginally increased the NK cell activity. The majority of the compounds were not able to reverse the multidrug resistance of mouse lymphoma cells. However, ginsenosides Rc, Rd and parishin C were able to moderately reduce the activity of the efflux pump. Parishin, parishin C and crude extract significantly enhanced the ADCC reaction.*

The combination of different *in vivo* and/or *in vitro* assay systems was recently recommended for enhancing the screening of bioactive compounds and for the quick and reliable deduction of pharmacological mechanisms. Previously, we found several pharmacological activities of naturally occurring compounds such as an inhibitory activity against skin tumor promotion (crocin and crocetin derivatives from *Crocus sativa*) (1) and membrane associated antitumor activity (ginsenosides, crocin and marihuana compounds) (2). In continuation of our investigation of bioactive natural resources, we did a preliminary survey of several glycosides with the use of a multidrug resistance assay system using lymphoma cells and leukocyte functions (3) and focused our interest in three kinds of glycosides: solasodine glycosides, ginsenosides and parishins. These three types of glycosides are important traditional Chinese medicines used as tonics which also possess some antitumor activities (4,5) although some of the derivates' biological and pharmacological activities are not yet known.

So far, no data has been reported in the literature on the effect on transmembrane signal and cancer cells. Therefore, we studied the effects of solamargine, solasonine, ginsenosides and parishin-related compounds on several leukocytes functions and on the reversal of multidrug resistance in lymphoma cells. The immunomodulating effects of the three different types of glycosides mentioned above were investigated on human peripheral blood mononuclear cells (PBM). The criteria we used were lymphocyte blast transformation activity, antibody dependent cellular cytotoxicity (ADCC) and the natural killing activity.

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Key Words: mdr-reversal, glycosides, immunomodulating effect, ADCC, NK reaction, blast transformation.

Materials and Methods

Compounds. Ginsenosides Rc, Rd, Re and Rg1 were isolated from the white ginseng by repeated column chromatography on silica gel as previously reported (6). They were directly identified with the authenic samples. Fruit of *S.khasianum* was obtained from the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University, Japan, and solasonine and solamargine were isolated as previously described (7) (Figure 1). Tuberous roots of *Gastrodia elata* were ground and extracted 3 times with 70% MeOH. The combined solution was evaporated under released pressure to give the crude extract. After partition of crude extract between H_2O and organic solvent, the aqueous layer was repeatedly purified on MCI gel CHP 20P using H_2O-MeOH mixture as a solvent to give parishin, parishin B and parishin C, respectively (Figure 2). All chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany), Pharmacia (Uppsala, Sweden) and Sigma-Aldrich Kft. (Budapest, Hungary). The compounds were dissolved in DMSO and final concentrations were 1.0 , 5.0 , 10 μg/ml, respectively in all triplicate test cultures. Both control cultures and test cultures contained DMSO in the same concentration.

Isolation of mononuclear cells. Human peripheral blood mononuclear (PBM) cells were isolated from healthy blood donors by Ficoll-Uromiro gradient centrifugation. The cells were washed and resuspended in

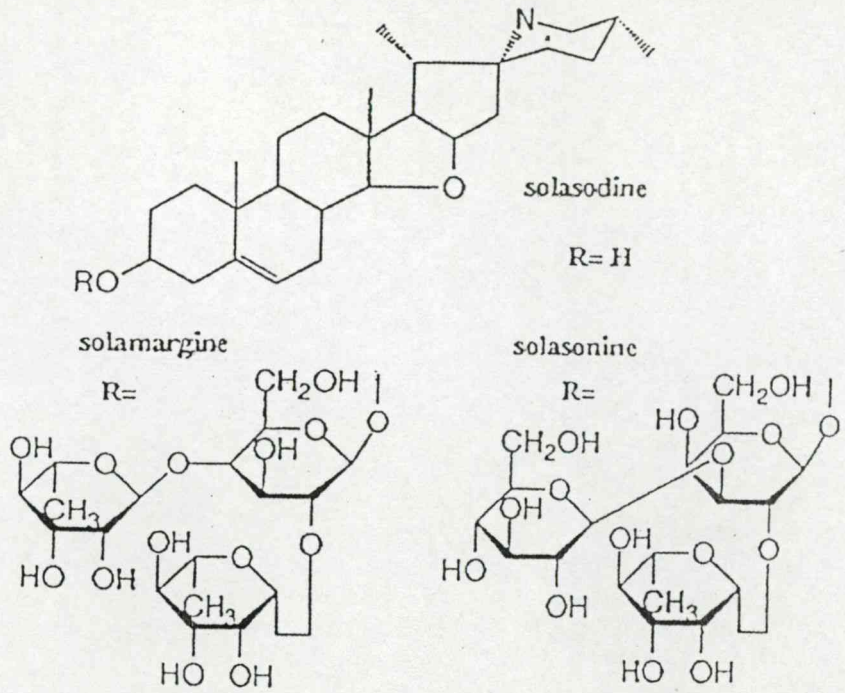

Figure 1. *The chemical structures of solasodine derivatives.*

RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. To provide monocyte-free (PBM-Mo-) samples, the freshly drawn heparinized blood was treated with carbonyl iron powder at 37°C for 5 minutes and then the lymphocytes were isolated on Ficoll-Uromiro gradient.
The viability of the cells with the drugs was determined by trypan blue indication after 4 hours incubation (8).

Lymphocyte blast transformation (T lymphocyte proliferation assays). Mononuclear cells were routinely cultured in flat-bottomed Greiner microtiter plates at $2x10^5$ cells (0.2 ml/well). The triplicate cultures containing the drugs were incubated in the presence or absence of mitogens: PHA 1:200 diluted stock solution, Con A 5 μg/ml in a CO_2 incubator for 72 hours. For the determination of lymphocyte DNA synthesis, 0.5 μCi ^{3}H-thymidine was added to all cultures for the last 5 hours and the cells were collected on GFC filter paper. The amount of radioactivity incorporated was determined in a liquid scintillation counter. The results were calculated as the difference in cpm between the incorporated activity of transformed cells and control cells (without mitogens) and the results were expressed as the % of control values (9).

Antibody dependent cellular cytotoxicity (ADCC) test. Human O Rh-positive red blood cells were used as a target and PBM Mo$^-$ cells as an effector in a 1:10 ratio. The reaction was mediated by red blood cell specific anti-D antibodies. The cultures were incubated at 37 °C for 16 hours and the amount of released ^{51}Cr in each supernatant was determined using a gamma counter. From the average of triplicate released values, the percentage of cytotoxicity was calculated according to the following formula (10):

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}$$

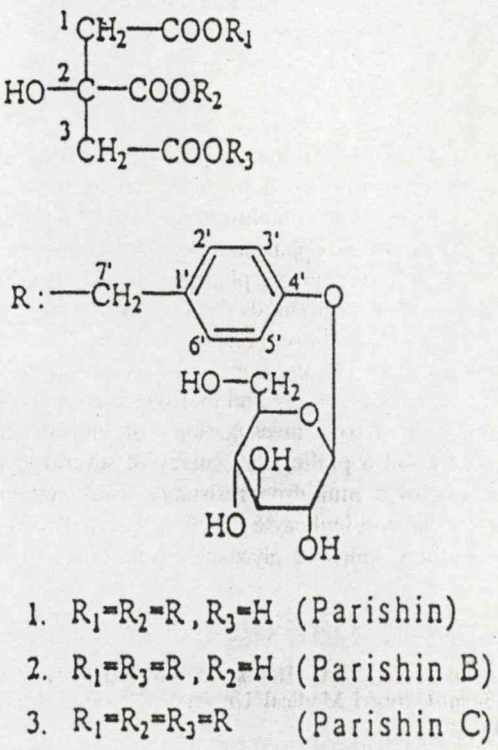

Figure 2. *The chemical structures of parishin derivatives.*

Spontaneous release indicated cultures without anti-D antibody. The results were expressed in percent of control cultures.

Natural killer cell activity (NK assay). ^{51}Cr-labelled K562 cells (used as the target) and PBM cells (used as the effector) were mixed in a ratio of 1:50. After incubation at 37°C for 4 hours, the amount of radioactivity released into the culture supernatants was determined. The results were expressed as the percentage of cytotoxicity, as described above (11).

Reversal of multidrug resistance. The L5178 mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus as previously described (12). MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of $2x10^6$/ml and resuspended in serum-free McCoy's 5A medium. The cells were then distributed in 0.5 ml aliquots to Eppendorf centrifuge tubes. The tested compounds were added in various concentrations (0.2, 20.0 µl) from the 1.0 mg/ml stock solutions and samples were incubated for 10 minutes at room temperature. Then 10 µl (5.2µM final concentration) indicator Rhodamine 123 was added to the samples and the cells were incubated for a further 20 minutes at 37 °C, washed twice and resuspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence of cell populations was measured by flow cytometry using a Beckton Dickinson FACSscan instrument. Verapamil was used as a positive control in the Rhodamine 123 exclusion experiments. The percentage of control mean fluorescence intensity was calculated for parental and mdr cell lines as compared to untreated cells. An activity ratio was calculated using the following equation (13) on the basis of measured fluorescence values:

$$R = \frac{\text{mdr treated / mdr control}}{\text{parental treated / parental control}}$$

Table I. *Reversal of multidrug resistance by solasodine glycosides, ginsenosides and parishins.*

Compounds	Conc. (µg/ml)	Fluorescence activity ratio
PAR		
MDR		
VERAPAMIL	5	4.36
SOLAMARGINE	2	1.26
	20	0.96
SOLASONINE	2	1.19
	20	0.6
GINSENOSIDE Rc	2	3.03
	20	2.33
GINSENOSIDE Rd	2	3.19
	20	4.18
GINSENOSIDE Re	2	1.83
	20	1.88
GINSENOSIDE Rgl	2	1.38
	20	1.57
PARISHIN	2	1.54
	20	2.93
PARISHIN B	2	1.49
	20	1.17
PARISHIN C	2	2.10
	20	1.34

Results

Cytotoxic effects of the compounds were determined by trypan blue exclusion tests. The biological effects were studied at non-toxic concentrations.

The cell viability was more than 95% with all compounds (data not given) and the reactions were calculated on viable cell number. The immunological effects of the compounds were tested on human peripheral blood mononuclear cells from different blood donors and all compounds were used in different concentrations. The data obtained from four different blood donors represented a single experiment and donor to donor variation was not significant. Some of the compounds, namely ginsenoside Rgl, ginsenoside Re, ginsenoside Rd and parishin, were able to reverse the multidrug resistance of tumor cells due to the inhibition of the mdr-1 mediated membrane efflux pump activity (Table I).

The inhibitory activity of the drugs tested on T cell proliferative assays was significantly stimulated by PHA and ConA in the presence of 1, 5 and 10 µg/ml doses. The exception was in the case of ginsenoside Rc and Rd, which had a considerable enhancing effect when tested on blast transformations and this effect exhibited dose-dependence (Figure 3).

Results of the ADCC reaction showed slight inhibitory activities in the case of 1 µg/ml concentration of the drugs (Figure 4). Parishin, parishin C and crude extract had a low dose- dependent enhancing effect.

The immunomodulating effect of solasonine, measured on NK cell activity, exhibited a dose-dependent inhibitory effect (Figure 5), but we could not detect any real immunomodulating effect in the case of the other compounds.

Discussion

In the present study we investigated the immunomodulating activities of three types of glycosides, dammarane-type glycosides: (ginsenosides), steroidal glycoalkaloids (solamargine and solasonine) and phenolic glycosides (parishin and related compounds). The crude drugs which contain the above glucosides have been used as traditional medicines, but fundamental questions remain unanswered concerning the nature of the compounds, and their roles in the protective mechanisms. Regarding their direct effects in immune reactions, we found that all compounds affected the immune functions of human peripheral blood mononuclear cells, but that effect was selective.

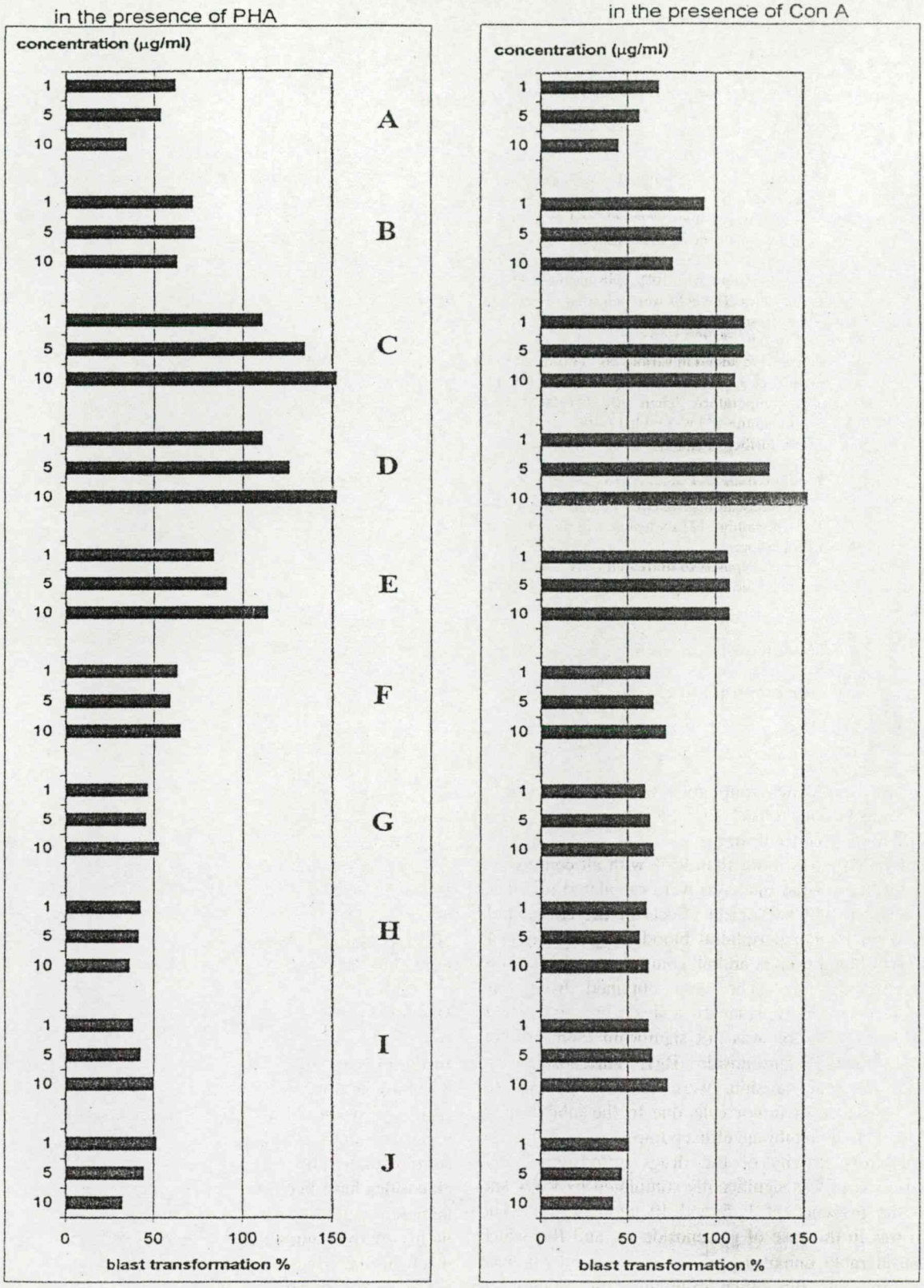

Figure 3. *A: solamargine, B: solasonine, C: ginsenoside Rc, D: ginsenosideRd, E: ginsenoside Re, F: ginsenoside Rgl, G: crude extract, H: parishin, I: parishin B, J: parishin C DNA synthesis of PHA- and Con A- stimulated lymphocytes were 25,300± 9/600 and 21,200±8,800 cpm, respectively.*

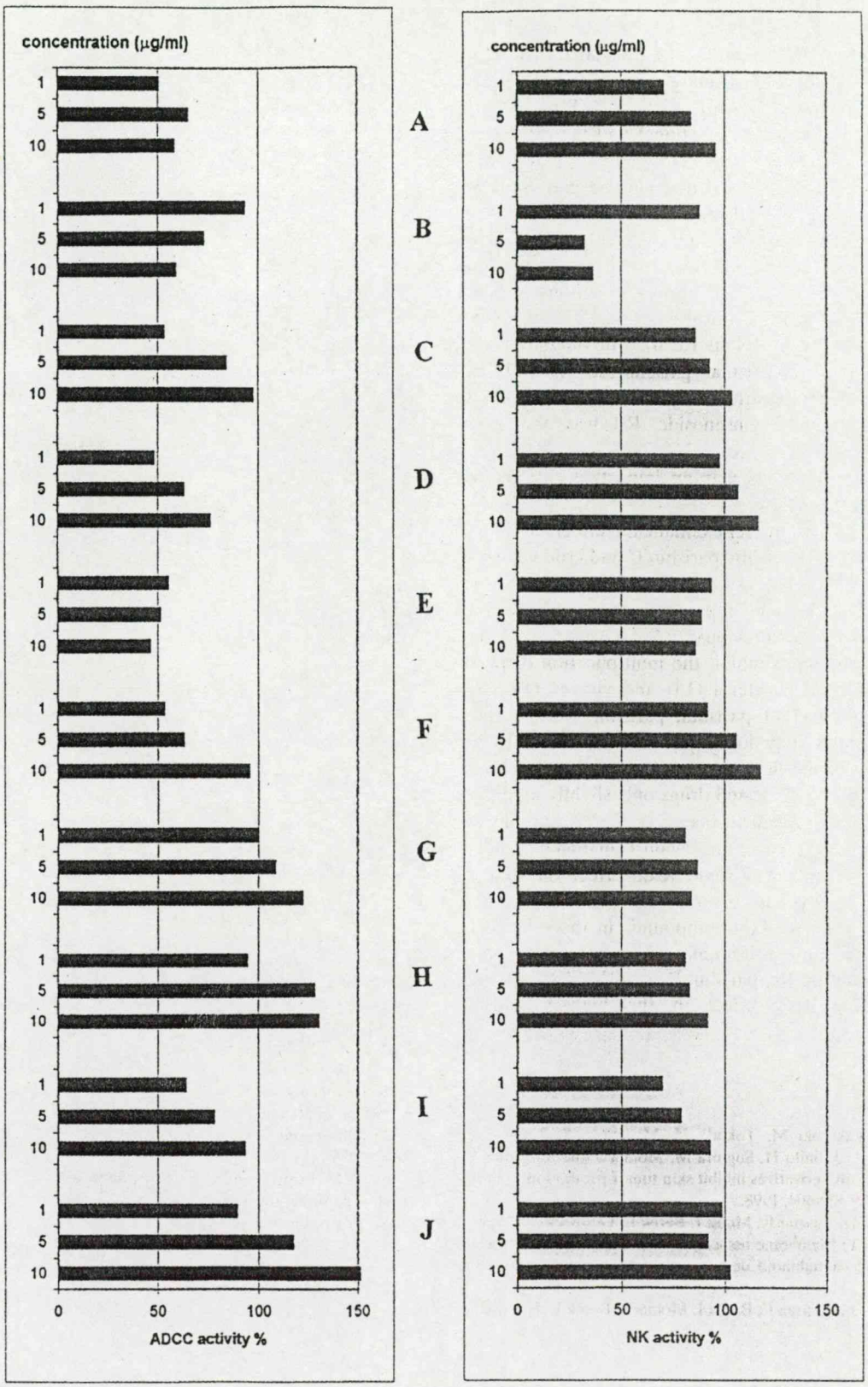

Figure 4. A: *solamargine, B: solasonine, C: ginsenoside Rc, D: ginsenoside Rd, E: ginsenosideRe, F: ginsenoside Rg1, G: crude extract, H: parishin, I: parishin B, J: parishin C The target-effector ratio was 1:10. The basic ADCC activity without DMSO was 43 %.*

Figure 5. A: *solamargine, B: solasonine, C: ginsenoside Rc, D: ginsenoside Rd, E: ginsenoside Re, F: ginsenoside Rgl, G: crudeextract, H: parishin, I: parishinB, J: parishin C. The target-effector ratio was 1:50. The basic NK cell activity without DMSO was 17 %.*

Solamargine and their related compounds were investigated in our laboratory for immunmodulatory functions and the reversal of multi drug resistance (MDR) in cancer (14). Solasonine and solamargine were the only drugs which inhibited all tested *in vitro* reactions (T and B cell proliferations, ADCC and NK reactions) and the effect was dose-dependent.

Previous studies have reported that ginseng is one of the most important Chinese medicines. It is used to enhance stamina, increase the capacity to cope with fatigue and physical stress and as a tonic for cancers and immune functions. The mechanisms of ginseng's actions remain unclear, although its pharmaceutical properties are attributed to the ginsenosides in the plant (5, 6). In our study the ginseng derivatives, in particular ginsenoside Rc and Rd, enhanced the T cell proliferative assays considerably. Among the studied compounds, ginsenoside Rd was the most effective compound with respect to MDR. The results suggest that these enhancing effects play an important role in the mechanism of ginseng action.

In the ADCC reaction, real enhancing effect could be detected in the case of parishin, parishin C and crude extract. The phenolic glycoside types of parishins were isolated from *Gastrodia elata*, which is an important, traditional Chinese medicine, used as a tonic. Previous studies have reported that some of these compounds inhibit the multiplication of plant and human pathogenic bacteria (15) and viruses (16, 17). These enhancing effect of parishin, parishin C and crude extract in the ADCC reaction are in accordance with the natural defense mechanism against infectious diseases (18).

In our experiments, the tested drugs only slightly modified the NK cell reaction, which plays a significant role in immunological defense reactions against malignancy. The majority of compounds had some toxic effect on cancer, while, some, were able to reverse the MDR of mouse lymphoma cells. The weakest compounds in these respects were solamargine and solasonine althougt solamargine, solasonine, ginsenoside Rc, parishin B , parishin C and crude extract had some toxic effect in the highest applied concentration.

References

1 Konoshima T, Takasaki M, Tokuda H, Morimoto S, Tanaka H, Kawate E, Xuan LJ, Saito H, Sugiura M, Molnár J and Shoyama Y: Crocin and crocetin derivatives inhibit skin tumor promotion in mice. Phytother Res *12:* 400-404, 1998.

2 Molnár J, Szabó D, Pusztai R, Mucsi I, Berek L, Ocsovszki I, Katawa E and Shoyama Y: Membrane associated antitumor effects of crocin-, ginsenoside- and cannabinoid derivatives. Anticancer Res *20:* 861-868, 2000.

3 Petri IB, Szekeres E, Varga E, Berek I, Molnár J, Berek L, Kawase M and Motohashi N: Immunomodulating activities on cellular cytotoxicity and the blast transformation of human leukocytes by 10-[n-(phtalimido)alkyl-2-substituted-10 H-phenothiazines and 1-(2-chloroethyl)-3-(2-substituted-10 H-phenothiazin-10-yl)alkyl-1-ureas. Anticancer Res *16:* 1247-1250, 1996.

4 Norman and Gillis C: Panax ginseng pharmacology: a nitric oxide link? Biochem. Pharmacology *54:* 1-8, 1997.

5 Tanaka H, Fukuda N and Shoyama Y: Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb1 and its characterization. *In:* Towards Natural Medicine Research in the 21st Century (Ageta H, Aimi N, Ebizuka Y, Fujita T and Honda G, eds). Amsterdam, Elsevier Science B.V., 249-259, 1998.

6 Tanaka H and Shoyama Y: Development of ELISA-analysis methods for the quantification of bioactive natural products in plants. phytomedicines and in humans or similar. Phytomedicine *5(5):* 397-415, 1998.

7 Mahato SB, Sahu NP, Ganguly AN, Kasai R and Tanaka O: Steroidal alkaloids from Solanum Khasianum: Application of ^{13}C NMR spectroscopy to their structural elucidation. Phytochemistry *19:* 2017-2020, 1980.

8 Molnár J, Mandi Y and Petri I: Immunomodulation activity of phenothiazines, benzo[*a*]phenothiazines and benz[c]acridines. Anticancer Res *13:* 439-442, 1993.

9 Molnár J, Petri IB, Berek I, Shoyama Y and Nidshioka I: The effects of cannabinoids and cannabispiro compounds on *E. coli* adhesion to tissue culture cells and on leukocyte functions *in vitro*. Acta Microbiol Acad Sci Hung *34:* 233-240, 1987.

10 Petri IB, Lorincz A and Berek I: (1986). Further investigation of nonspecific substance in anti-Rh(D) preparation. Vox Sang *51:* 287-291, 1986.

11 Petri IB, Szekeres E, Berek I, Molnár J, Sakagami H, and Motohashi N: (1993). Effect of Benzo(a)phenothiazines on Natural Killer cell activity of peripheral blood mononuclear cells. Anticancer Res *13:* 2273-2276, 1993.

12 Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV, and Willigham MC: A retrovirus carrying MDR1 cDNA confers multiple drug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Nat Acad Sci (Wash.) *85:* 4486-4490, 1988.

13 Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S: The effect of ion chanel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int J Cancer *54:* 456-461, 1993.

14 Szabo D and Molnár J: The role of stereoselectivity of chemosensitizers in the reversal of of multidrug resistance of mouse lymphoma cells. Anticancer Res *18:* 3039-3044, 1998.

15 Shoyama Y, Matsumoto M and Nishioka I: (1987). Phenolic glycosides from deseased roots of *Rehmannia glutinosa var.purpurea.* Phytochemistry *26:* 983-986, 1987.

16 Pierpoint WS, Ireland RJ and Carpenter JM: Modification of proteins during the oxidation of leaf phenols: Reaction of potato virus x with chlorogenoquine. Phytochemistry *16:* 29-34, 1997.

17 Shoyama Y, Matsumoto M and Nishioka I: Four caffeoyl glycosides from callus tissue of Rehmannia glutinosa. Phytochemistry *25:* 1633-1636, 1986.

18 Legrand M, Fritig B and Hirth L: (1976). Enzymes of the phenylpropanoid pathway and the necrotic reaction of hypersensitive tobacco mosaic virus. Phytochemistry *15:* 1353-59.

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Effect of Some New 3-Benzazepines on Plasmid DNA, *mdr* P-glycoprotein and Reverse Transcriptase of Leukaemia

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Abstract

Two 3-benzazepines ([5], [10]) were able to form complex with replicative form of plasmid DNA. The multidrug resistance (*mdr*) P-glycoprotein efflux pump of mouse lymphoma cells was inhibited by three compounds ([5], [8], [10]). The inhibitory effects of compounds on reverse transcriptase (RT) of Moloney leukaemia had shown a great variety, however, the most effective compounds were [7], [8] and [9].

Introduction

Antibiotic resistance of bacteria is encoded by extrachromosomal DNA, called plasmids. There are plasmids encoding ATP binding cassette (ABC) transporter protein which have a great extent of similarity with a P-glycoprotein (P-gp) responsible for multidrug resistance (*mdr*) of cancer cells (Endicott and Ling, 1989). The emerging drug resistance is known

Abbreviations: 7,8-Dimethoxy-2-methyl-3-methanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [1], 7,8-dimethoxy-2-methyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [2], 3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [3], 7,8-dimethoxy-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [4], 7,8-dimethoxy-2-isopropyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [5], 7,8-dimethoxy-2-phenyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [6], 7,8-dihydroxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [7], 7,8-dihydroxy-3-methyl-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [8], 7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine [9], 7,8-dimethoxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [10], dopamine [DA, 11]; norepinephrine [NE. 12].

among viruses, bacteria, protozoa and cancer cells including drug resistant retroviruses such as human immunodeficiency virus (HIV) (Hamada and Tsuruo, 1986; Zamora *et al.*, 1988; Kovács *et al.*, 1991; Schäfer *et al.*, 1993; Swartz, 1994; Taylor *et al.*, 1994; Artico *et al.*, 1996; Ecker *et al.*, 1996; Hewlett *et al.*, 1997; Suzuki *et al.*, 1997).

In addition, retroviruses can exist in an autonomous state and behave like plasmids-before the integration into host cell chromosomal DNA.

There are evidences that antiplasmid compounds can form a complex with plasmid DNA topoisomerase and *mdr* P-gp, resulting in the reversal of resistance. However, the majority of compounds do not block replication of HIV retrovirus although the reverse transcriptase (RT) and integrase were considered potential targets for inhibition of retrovirus replication. The search for novel agents has led to the synthesis of some rare phenothiazines (Motohashi *et al.*, 1996) and 3-benzazepines (Kawase *et al.*, 1997; Kawase *et al.*, 1998b).

To identify the standard features of 3-benzazepines required for the biological activities, the tetrahydro-3-benzazepines were subjected to interact with *E. coli* plasmid DNA, *mdr* P-gp of lymphoma cells and RT of Moloney leukaemia in model experiments.

Materials and Methods

Chemicals-The following six 2,3,4,5-tetrahydro-3-benzazepinones [**1-6**] and three 2,3,4,5-tetrahydro-1*H*-3-benzazepines [**7, 8, 10**] were newly synthesized as recently published (Kawase *et al.*, 1997; Kawase *et al.*, 1998b). Compound [**9**] was synthesized, vide literature (Pecherer *et al.*, 1971). [**1**] (m.w. 313.38, mp 165-166°C), [**2**] (m.w. 367.34, mp 102-103°C), [**3**] (m.w. 293.26, mp 76°C), [**4**] (m.w. 353.32, mp 158-160°C), [**5**] (m.w. 395.40, crystals), [**6**] (m.w. 429.42, mp 175-178°C), [**7**] (m.w. 328.13, mp 223°C (dec)), [**8**] (m.w. 297.70, mp 215°C (dec)), [**9**] (m.w. 206.12, mp 241°C (dec)), [**10**] (m.w. 275.24, oil).

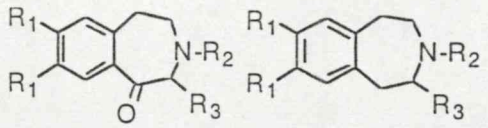
DA hydrochloride [**11**] was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NE [**12**] and rhodamine 123 hydrate (R123, lot#28394-0) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) (Table 1).

DA [**11**]) and NE [**12**]) were used as controls and the compounds were dissolved in 20% dimethylsulfoxide (DMSO) solution.

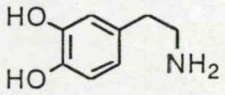
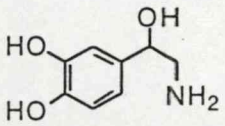
Bacterial strains.

E. coli K12 pBR 322 bacterial strain was applied for isolation of plasmid DNA by agarose gel-electrophoresis (Fig. 1).

Table 1. Structures of 2,3,4,5-tetrahydro-1*H*-3-benzazepines [**1-10**] and two catechols [**11,12**] used in this study[a].



[**1-6**] [**7-10**]

Compound	R_1	R_2	R_3
[**1**]	MeO	Ms	Me
[**2**]	MeO	Tf	Me
[**3**]	H	Tf	H
[**4**]	MeO	Tf	H
[**5**]	MeO	Tf	Me_2CH
[**6**]	MeO	Tf	Ph
[**7**]	HO	H	CF_3
[**8**]	HO	Me	CF_3
[**9**]	HO	H	H
[**10**]	MeO	H	CF_3
DA [**11**]			
NE [**12**]			

a) abbreviations. Me: CH_3 Ms: SO_2CH_3; Tf: SO_2CF_3.

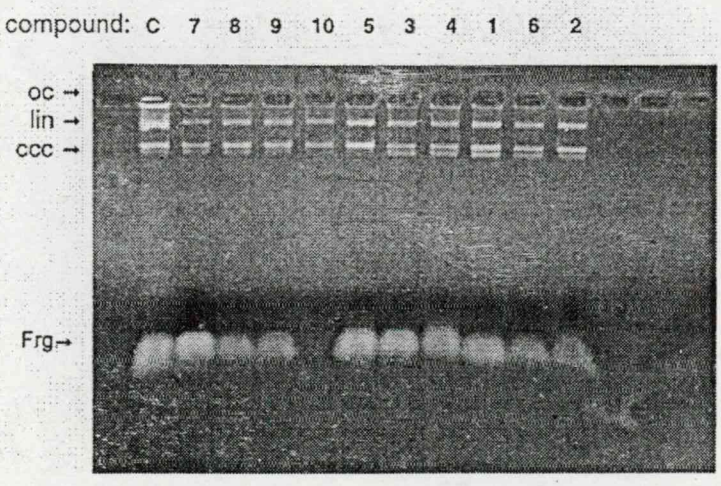


Fig. 1. Effect of tetrahydrobenzazepines on pBR322 plasmid DNA from *E. coli.* (C-control; ccc-covalently closed circular form; oc-open circular form; lin-linear form; and Frg-fragment of DNA

Antiretroviral effect. Moloney murine leukaemia virus (M-MuLV) RT assay.

Trifluoperazine-metal coordination complexes and 3′-azido-3′-deoxythymidine triphosphate (AZT-TP) as a control were assayed for their ability to inhibit M-MuLV RT (New England BioLabs). The assay is based on following: the poly-adenosine phosphate $(rA)_n$, oligo deoxythymidine $(dT)_{12\text{-}18}$ (New England BioLabs) directed incorporation of deoxythymidine triphosphate (dTTP) (Amsterdam) into complementary DNA (cDNA). The 10 × RT buffer contains 500 m*M* Tris-HCl (*p*H 8.3), 80 m*M* $MgCl_2$, 300 m*M* KCl, and 100 m*M* dithiothreitol (DTT). In all experiments, the final volume of the reaction assay was 20 μL. This contained water, 2 μL of 10 × RT buffer, 20 μg/mL template-primer, 5 μ*M* dTTP precursor (New England BioLabs), 0.2 μCi tritiated precursor, the compounds to be tested (administrated into the medium before adding the enzyme) and 5 units (U) RT initiating the reaction. This procedure was followed by incubation for 40 min at 37°C. 15 μL of the mixture was then transferred to Whatman DE81 filter paper disc, washed by 5% disodium-hydrophosphate buffer (3 × 3 min), water, then 96% ethanol, and after drying and putting into 5 mL scintillation cocktail (OptiPhase HiSafe 3′, Wallac), the radioactivity was measured by Packard Tri-Carb 2200 CE liquid scintillation counter. The residual enzymatic activities were compared to the control (no drug added). The IC_{50} of AZT-AT was 0.12 μ*M* in our experiments.

Cell and fluorescence uptake, mdr reversal effect.

The L5178 mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus as previosuly described (Aszalos *et al.*, 1995). MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A tissue culture medium with 10% heat-inactivated horse serum, L-glutamine and 2,3,4,5-tetrahydro-1*H*-3-benzazepines [**1-10**]. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquote to Eppendorf centrifuge tubes. Then, the tested compounds were added in various concentrations (0.2-20.0 μL) of the 1.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μ*M* final concentration) indicator of rhodamine 123 were added to the samples and the cells were incubated for further 20 min at 37°C, washed twice and resuspended on 0.5 mL phosphate-buffered saline

(PBS) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the rhodamine 123 exclusion experiments (Weaver *et al.*, 1993). The percentage of control mean fluorescence intensity was calculated for the following equation on the basis of measured fluorescence values.

$$R = \frac{mdr \text{ treated}/mdr \text{ control}}{\text{parential treated/parental control}}$$

Complex formation between benzazepines and plasmid DNA

The *E. coli* pBR322 strain was grown in 5 mL yeast-extract-tryptone broth (YTB) in presence of tetracycline (100 µg/mL) and ampicillin (25 µg/mL) at 37°C for 16 hours. The overnight culture was distributed into 1 mL aliquote in Eppendorf tubes. Benzazepines were added to the plasmid containing bacterial suspension in 200 µg/mL final concentration, the samples were centrifuged for 5 min (3000 rpm) and the supernatant was discarded. The pellet was resuspended in 100 µL of Sol I (1*M* Tris-HCl, 0.5 *M* glucose, 0.25 *M* ethylenediaminetetraacetic acid (EDTA)), homogenized in vortex, and incubated at room temperature for 5 min. Then, 200 µL of Sol II (10 N NaOH, 20% sodium dodecyl sulfate (SDS)) was added to the bacterial suspensions, homogenized and incubated in water bath (0°C) for 5 min. Then, 150 µL of ice cold 5 *M* potassium acetate was added to the samples. After mixing, samples were incubated further for 5 min at 0°C, then centrifuged for 10 min (3000 rpm). The supernatant was treated with 200 µL phenol solution and 200 µL of chloroform: isoamyl alcohol (1:1). The samples were centrifuged for 1 min (3000 rpm) and the supernatants were precipitated with 96% ethanol and incubated at room temperature for 5 min and then precipitated plasmid DNA was centrifuged for 5 min (3000 rpm). The supernatant was discarded and the plasmid DNA was washed with 70% ethanol. Then, ethanol was discarded and the samples were dried at 37°C. The extracted plasmid DNA was dissolved in 20 µL Tris-EDTA (TE)-buffer containing RNAse (20 µL/mL, Sigma). 10 µL of the RNAse treated plasmid DNA solution were applied to 1% agarose gel (with 0.5 µg/mL ethidium bromide). Agarose gel electrophoresis was performed for 20 min (200 V). The various forms of plasmid DNA were detected under UV lamp (Fluo-link).

Results and Discussion

In the control sample [C], the covalently closed circular (ccc) form of plasmid DNA runs faster than the linear (lin) form and the two conformations can be seen near to each other (Fig. 1). The open circular

(oc) form ran slower, relatively far from linear (lin) form. In case of 2,3,4,5-tetrahydro-1*H*-3-benzazepine treated plasmid-containing cells, the oc form can be found in each sample. Similar situation exists with lin form. However, compounds ([**5**], [**10**]) were able to form complexes with the ccc form of plasmid DNA. The results showed an evidence for the selective complex formation of two active benzazepines ([**5**], [**10**]) with the superhelical form of plasmid DNA, meaning that this biological effect is dependent on the substituents of the benzazepine molecule.

The ccc form of plasmid DNA is maintained by bacterial gyrase (topoisomerase) which can be inhibited by benzazepines. Theoretically, it is also possible that a simple interaction of two benzazepines ([**5**], [**10**]) leads to the unwinding of ccc form into the oc form, which is due to a single nick introduced into one strand.

Two compounds ([**5**], [**10**]) selectively inactivate the replicative form of plasmid DNA that encode the antibiotic resistance (tetracycline (Tc) and ampicillin (Ap)) in bacteria. The DA [**11**] and NE [**12**] had no effects (data not shown).

When the substituted benzazepines were tested on the P-glycoprotein efflux-pump of tumour cells, only a few compounds were active (Table 2).

Mdr reversal on tumour cells

The *mdr* reversing effects of benzazepines [**1-10**] by their chemical structures were compared to that of verapamil, using a mouse leukaemia cell line (L-5178 cells). The effects were measured by fluorescence ratio between treated and untreated group cells. Compound [**8**] has the highest activity on *mdr* reversal (Fluorescence activity ratio 8.38) among 12 compounds used in this research and compound [**8**] was 2-fold more potent than verapamil (fluorescence activity ratio 4.18). Then, compound [**8**] might be an anti-*mdr* inducing agent of great interest, because the affinity of [**8**] to two dopamine D_1 and D_2 receptors was reduced by introduction of trifluoromethyl (CF_3) group at 2nd position of benzazepine ring (Kawase *et al.*, 1998b).

We have reported that benzazepines [**7**], [**8**] and [**9**] induced apoptic cell death in human promyelocytic leukaemia HL-60 cells and the cytotoxic activities of [**7**]-[**9**] were 1.3, 1.3 and 2.0 times higher than that of DA [**11**], respectively. Additionally, it is found that apoptosis induced by these benzazepines [**7**]-[**9**] is coupled with their radical generation (Kawase *et al.*, 1998a).

The retroviruses have a key enzyme, which called RT that is responsible for maintaining the virus multiplication, the transformation of

RNA encoded information into DNA. This intermediate stage of retroviral replication was effected by three compounds ([7], [8], [9],) (Table 3) (Figure 1).

The results obtained show a chemical structure-dependent effect on RT again. The active benzazepines inhibit the RNA-directed DNA synthesis, however, the majority of the derivatives did not show remarkable

Table 2. The effect of 2,3,4,5-tetrahydo-1*H*-3-benzazepines [**1-10**] and two analogues [**11, 12**] on lymphoma 5178 cells with multidrug resistance.

Compound [No.]	Concentration (μg/mL)	Forward scatter height [cell size ratio]	Side scatter height [granulation of cell ratio]	Fluorescence one height[a] value			Fluorescence activity ratio[c]
				x[b]	$y=\frac{x}{256}$	$\log(y)=\log_{10}\frac{x}{256}$	
par[e]	control	504.86	184.75	775.68	3.03	1060.98	49.98
mdr+R123[f]	control	552.95	211.52	340.48	1.33	21.23	1.00
(±)verapamil[d]	5	545.74	216.62	499.20	1.95	88.72	4.18
Benzazepines:							
[1]	5	473.30	188.91	253.44	0.99	9.85	0.46
[2]	5	425.16	205.05	325.12	1.27	18.69	0.88
[3]	5	494.57	193.54	284.16	1.11	12.97	0.61
[4]	5	467.29	188.79	309.76	1.21	16.39	0.77
[5]	5	491.72	190.79	422.40	1.65	44.77	2:11
[6]	5	507.50	197.30	291.84	1.14	13.89	0.65
[7]	5	469.78	187.09	314.88	1.23	17.01	0.80
[8]	5	447.77	191.53	576.00	2.25	177.91	8.38
[9]	5	477.28	189.43	309.76	1.21	16.12	0.76
[10]	5	507.71	198.41	373.76	1.46	28.68	1.35
Dopamines:							
DA [11]	2	579.02	251.38	343.04	1.34	21.77	0.84
	20	571.20	251.12	350.72	1.37	23.47	0.93
NE [12]	2	569.10	256.09	340.48	1.33	21.32	0.82
	20	564.14	254.11	330.24	1.29	19.53	0.80

a) Ref: Kessel,1989; Weber *et al.*, 1994.
b) x: Measured fluorescence value at linear scale [mg, uptake of R123].
c) The R-123 accumulation was calculated from fluorescence of one height value using 1st equation

$$\log(y)=\log_{10}\frac{x}{256}$$

then the fluorescence activity ratios were calculated according to the formula given below;

$$\text{Ratio} = \frac{(mdr \text{ treated}/mdr \text{ control})}{(\text{parental treated}/\text{parental control})}$$

d) (±)verapamil: a control for *mdr* reversal
e) par: parental without multidrug resistant gene.
f) *mdr*: parental with multidrug resistant gene.

Table 3. Inhibition of Moloney murine leukaemia virus rever transcriptase by 2,3,4,5-tetrahydro-1*H*-3-benzazepines [1-10] In concentration ($10^{-5}M$).

Compound (No.)	Concentration (μg/mL)	Activity of cDNA (DPM)[a)]	Activity in percentage of DMSO control (%)
DMSO (control) 0.5 μL DMSO/ 20 μL assay		67401 54887 50297 64784	100
+ Control		83245 57408	-
[1]	50	39352 60832	88.2
[2]	50	49649 54104	89.1
[3]	50	48949 45591	80.4
[4]	50	63098 66862	112.6
[5]	50	56214 58153	98.7
[6]	50	31249 52950	72.1
[7]	50	23728 13754	32.6
[8]	50	17873 17305	29.5
[9]	50	15536 10126	21.8
[10]	50	60242 56805	102.6

[a]DPM: decomposition per minute [alternative to count per minute (cpm) values].

effects in the system. These derivatives possibly might modify the enzyme activity by binding to benzazepine binding sites on RT-enzyme.

References

Artico M, Silvestri R, Pagnozzi E, Stefancich G, Massa S, Loi AG, Putzolu M, Corrias S, Spiga MG and Colla PL. 1996. 5*H*-Pyrrolo[1,2-*b*][1,2,5]benzothiadiazepines (PBTDs): A novel class of non-nucleoside reverse transcriptase inhibitors. *Bioorganic Medicinal Chem*, 4: 837-850.

Aszalos A, Pine PS, Pandey R and Gottesman MM. 1995. Behavior of N-acylated daunorubicins in MDR1 gene transfected and parental cells. *Biochem Pharmacol*, 50: 889-892.

Ecker G, Chiba P, Hitzler M, Schmid D, Visser K, Cordes HP, Csöllei J, Seydel JK and Schaper KJ. 1996. Structure-activity relationship studies on benzofuran analogs of propafenone-type modulators of tumor cell multidrug resistance. *J Med Chem*, 39: 4767-4774.

Endicott JA and Ling V. 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem*, 58: 137-171.

Hamada H and Tsuruo T. 1986. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant-tumor cells as revealed by monoclonal antibiotics. *Proc Natl Acad Sci USA* 83: 7785-7789.

Hewlett I, Lee S, Molnár J, Foldeak S, Pine PS Weaver JL and Aszalos A. 1997. Inhibition of HIV infection of H9 cells by chlorpromazine derivatives. *J Acquired Immune Deficiency Syndrom Human Retroviolog*, 15: 16-20.

Kawase M, Motohashi N, Niwa M and Nozaki M. 1997. Use of the triflamide group for Friedel-Crafts acylation of *N*-(β-phenethyl)amino acids to 3-benzazepine derivatives. *Heterocycles*, 45: 1121-1129.

Kawase M, Motohashi N, Chakrabarty AN, Dastidar SG, Kurihara T, Inagaki M, Sakagami H, Satoh K, Saito S and Molnár J. 1998a. Cytotoxic activity and radical intensity of 3-benzazepine derivatives. *In*: Chakrabarty AN, Molnár J, Dastidar SG and Motohashi N (eds). *Non Antibiotics*, NISCOM, New Delhi, India.

Kawase M, Niwa M, Nozaki and Motohashi N. 1998b. Synthesis of 2-trifluoromethyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine derivatives. *Heterocycles*, 48: 555-560.

Kessel D: Exploring multidrug resistance using rhodamine 123. 1989. *Cancer Commun*, 1: 145-149.

Kovács T, Parkanyi L, Pelczer I, Cervantes-Lee F, Pannel KH and Torrence PF. 1991. Solid-state and solution conformation of 3'-amino-3'-deoxythymidine, precursor to a noncompetitive inhibitor of HIV-1 reverse transcriptase. *J Med Chem*, 34: 2595-2600.

Motohashi N, Kawase M, Kurihara T, Hevér A, Nagy S, Tanaka M and Molnár J. 1996. Synthesis and antitumor activities of 1-(2-chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkylureas as potent anticancer agents. *Anticancer Res*, 16: 2525-2532.

Pecherer B, Sunbury RC, Brossi A. 1971. The synthesis of some 7- and 7,8-substituted 2,3,4,5-tetrahydro-1*H*-3-benzazepines. *J Hetercocyclic Chem*, 8: 779-783.

Schäfer W, Friebe WG, Leinert H, Mertens A, Poll T, Saal WV, Zilch H, Nuber B and Zieger ML. 1993. Non-nucleoside inhibitors of HIV-1 reverse transcriptase: Molecular modeling and X-ray structure investigations. *J Med Chem*, 36: 726-732.

Suzuki T, Fukazawa N, San-nohe K, Sato W and Yano O. 1997. Structure-activity relationship of newly synthesized quinoline derivatives for reversal of multidrug resistance in cancer. *J Med Chen*, 40: 2047-2052.

Swartz MN. 1994. Hospital-acquired infections:diseases with increasingly limited therapies. *Proc Natl Acad Sci USA* 91: 2420-2427.

Taylor PB, Culp JS, Debouck C, Johnson RK, Patil AD, Woolf DJ, Brooks I and Hertzberg RP. 1994. Kinetic and mutational analysis of human immunodeficiency virus Type 1 reverse transcriptase inhibition by inophyllums, a novel class of non-nucleoside inhibitors. *J Biol Chem*, 269: 6325-6331.

Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S and Aszalos A. 1993. The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. *Int J Cancer*, 54: 456-461.

Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J and Rosenberg SA: 1994. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. *Cancer Res*, 54: 1766-1771.

Zamora JM, Pearce HL and Beck WT. 1988. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol*, 33: 454-462.